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<p>(54) Title: RECEPTORS FOR FIBROBLAST GROWTH FACTORS</p> <p>(57) Abstract</p> <p>A fibroblast growth factor (FGF) receptor including a basic fibroblast growth factor receptor has been purified. Various forms have been identified including soluble forms lacking any transmembrane segment. DNA sequences encoding full-length fibroblast growth factor receptors and polypeptides comprising a portion of an FGF-R ligand-binding domain have been isolated and sequenced. These DNAs include DNAs encoding for a basic FGF-R and a human FGF-R and are operably linked to control sequences and expressed in a culture of a compatible host transformed, transfected or electroporated by a cloning vehicle containing the DNA sequence. The invention also comprises antibodies to the receptor, methods of synthesizing the growth factor receptor proteins, methods for providing analogs of the fibroblast growth factor receptors. Methods for evaluating compositions which promote or inhibit fibroblastic growth factors and compositions which are agonistic or antagonistic to fibroblast growth factor receptors are also provided. Diagnostic and therapeutic uses are described.</p>		

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RECEPTORS FOR FIBROBLAST GROWTH FACTORS

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This application is a continuation-in-part application of commonly assigned patent application U.S.S.N. 07/377,003 filed on July 6, 1989, which is hereby incorporated herein by reference.

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Field of the Invention

The present invention relates to receptors for growth factors, specifically to the fibroblast growth factor receptor (FGF-R). More particularly, it provides various purified fibroblast growth factor receptor proteins, nucleic acids encoding the receptor proteins, methods for the production of purified FGF-R proteins, proteins made by these methods, antibodies against these proteins, and diagnostic and therapeutic uses of these various reagents.

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BACKGROUND OF THE INVENTION

Polypeptide growth factors are mitogens that act on cells by specifically binding to receptors situated at the plasma membrane. These receptors usually have three major identifiable regions. The first is an extracellular region which contains the domain that binds the polypeptide growth factor (i.e. the ligand-binding domain). The second region is a transmembrane region and the third is an intracellular region. Many of these receptors contain a tyrosine kinase domain in the intracellular region.

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The fibroblast growth factor receptor (FGF-R) proteins bind to a family of related growth factor ligands, the fibroblast growth factor (FGF) family. This family of growth

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factors are characterized by amino acid sequence homology, heparin-binding avidity, the ability to promote angiogenesis and mitogenic activity toward cells of epithelial, mesenchymal and neural origin.

5 The FGF family includes the following seven known FGFs:

(1, 2) acidic FGF (aFGF) and basic FGF (bFGF) (D. Gospodarowicz et al., Mol. Cell. Endocrinol., 46:107 (1986));

10 (3) the int-2 gene product (R. Moore et al., EMBO. J., 5:919 (1986));

(4) the hst gene product or Kaposi's sarcoma FGF (K.J. Anderson et al. Nature, 332:360 (1988); M. Taira et al., Proc. Natl. Acad. Sci. USA, 84:2980 (1987));

15 (5) FGF-5 (X. Zhan et al., Mol. Cell. Biol., 8:3487 (1988)); and

(6) keratinocyte growth factor (J.S. Rubin et al., Proc. Natl. Acad. Sci. USA, 86:802 (1989)).

20 (7) FGF-6 (I. Marics, et al., Oncogene 3:335 (1989)).

The actions of acidic and basic FGF are mediated through binding to high affinity cell surface receptors of approximately 145 and 125 kDa (G. Neufeld and D. Gospodarowicz, J. Biol. Chem., 261:5631 (1986)).

25 The reference of Imamura et al., "Purification of Basic FGF Receptors from Rat Brain," Biochem. Biophys. Res. Communications, 155:583 (September 15, 1988) discloses the purification of nanogram amounts of a basic FGF receptor (bFGF-R) from rat brain.

30 While genes encoding a number of growth factor receptors have been molecularly cloned (e.g., mouse PDGF receptor, Yarden et al., Nature, 323:226 (1986)), no clone has previously been identified as encoding a fibroblast growth factor receptor (FGF-R). Using antiphosphotyrosine antibodies
35 to screen λ gt11 cDNA expression libraries, a 2.5 kilobase cDNA encoding a novel tyrosine kinase gene, designated bek (bacterially expressed kinase), was isolated from a mouse liver cDNA library. (S. Kornbluth et al., "Novel Tyrosine Kinase

Identified by Phosphotyrosine Antibody Screening of cDNA Libraries", Mol. Cell. Biol. No. 8, 5541 (1988)). The bek sequence did not contain a transmembrane region and therefore could not be identified as a growth factor receptor. Another
5 protein tyrosine kinase gene designated flg (fms-like-gene) was isolated from a human endothelial cell cDNA library by hybridization under relaxed stringency with a v-fms oncogene probe. (M. Ruta et al., "A Novel Protein Tyrosine Kinase Gene
10 Whose Expression is Modulated During Endothelial Cell Differentiation", Oncogene, 3:9 (1988)). Those authors could not identify a transmembrane region in their isolated sequence and therefore hypothesized that flg encodes a cytoplasmic tyrosine kinase.

The purified and cloned chicken bFGF and human bFGF
15 receptors of this invention have amino acid sequence similarity with the bek and flg clones in the regions which have been isolated. However, both the bek and flg sequences reported were incomplete and there was no recognition of their function as FGF binding receptors. Moreover, the prior reports failed
20 to recognize many of the structural and functional features described in the present invention.

Members of the FGF family appear to have roles in tissue development, tissue repair, maintenance of neurons and in the pathogenesis of disease. Aberrant expression of FGF may
25 cause cell transformation by an autocrine mechanism. Moreover, FGFs may enhance tumor growth and invasiveness by stimulating blood vessel growth in the tumor or by inducing production of proteins such as plasminogen activator. However, identification of the components involved and understanding of
30 the mechanisms and interactions involved remain woefully incomplete.

Purified FGF receptors and fragments, and isolated DNA sequences encoding defined FGF receptors and defined
35 fragments (e.g., the ligand-binding domain) will greatly accelerate the understanding of fibroblast growth factor functions. Antibodies against specific and defined regions of the FGF receptor also become available. These reagents will find both diagnostic and therapeutic uses in the aforementioned

processes. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

5 The present invention provides purified fibroblast growth factor receptor (FGF-R) proteins, nucleic acids encoding FGF-R proteins, methods for the production of purified FGF-R proteins, purified proteins made by these methods, antibodies against these proteins and fragments, and diagnostic and
10 therapeutic uses of these reagents. Notably, the present invention provides soluble and secreted forms of the receptors exhibiting an unusual receptor structure.

 The present invention provides a method for modifying in vivo a fibroblast growth factor receptor modulated activity
15 comprising administering to a patient an amount of a fibroblast growth factor receptor blocking agent effective to inhibit fibroblast growth factor binding to said fibroblast growth factor receptor. Typically, the agent will be a fragment of a human fibroblast growth factor receptor, e.g., a fragment
20 produced in a cell transformed with a nucleic acid containing at least about 15 bases of a sequence selected from the group consisting of:

- a) a DNA sequence in Figures 3 or 4;
- b) a sequence encoding a polypeptide of Figures 3, 4 or
25 7; and
- c) a sequence substantially homologous to a sequence of Figures 3 or 4.

The fragment will often be a fibroblast growth factor receptor extracellular domain without a tyrosine kinase region.

30 Alternatively, a method is provided for inhibiting binding between a fibroblast growth factor and a fibroblast growth factor receptor in a solution. This method will contain a step of combining an FGF-R peptide, e.g., a peptide homologous in sequence to a sequence described in Figures 3, 4
35 or 7 to a solution or medium containing fibroblast growth factor and fibroblast growth factor receptor, usually native fibroblast growth factor receptor. Such methods will be useful in vitro, after employing labeled FGF-R peptide in assay

procedures.

Compositions containing a soluble FGF-R polypeptide having between about five and two hundred contiguous amino acids from a human FGF-R extracellular domain are described.

5 In one embodiment, the polypeptide contains at least about 80 amino acids from residues 1 to 287 of a human fibroblast growth factor receptor of Figure 7 or an IgII or IgIII domain, or both. In alternative embodiments, the IgII domain will have about 7 contiguous amino acids from residues 85 to 141 of a
10 human sequence of Figure 7 or may contain a carboxy-terminal sequence substantially homologous to the 79 amino acid sequence from residues 222 to 300 of a soluble human protein of Figure 7. Particularly preferred polypeptides consist essentially of the h4 or h5 sequences (Figure 7).

15 A further aspect of the invention is a fibroblast growth factor receptor composition containing a substantially pure polypeptide of less than about 85 KDa comprising a fibroblast growth factor-binding domain. The polypeptide may be soluble or may specifically possess a signal segment, an IgI
20 segment, an acidic segment, an IgII segment, an IgIII segment, an IgIIIT segment, or a transmembrane segment. Preferred embodiments will be homologous to a sequence described in Figures 3, 4 or 7 or will include at least about 30 amino acids of each of both IgII and IgIII domains. The polypeptide can be
25 one polypeptide chain in a multi-chain complex of proteins. A chicken fibroblast growth factor receptor is one preferred embodiment.

The present invention embraces isolated nucleic acids encoding human fibroblast growth factor receptor proteins which
30 substantially lack an intracellular domain. Such a nucleic acid will usually exhibit a sequence homologous to an IgII domain described in Figure 7, or may include a substantially full length IgII domain. The nucleic acid will usually also have a signal segment, an IgI segment, an acidic segment, an
35 IgIII segment, an IgIIIT segment, a transmembrane segment, or a tyrosine kinase segment, and will preferably correspond to a sequence described in Figures 3, 4 or 9. A particularly preferred embodiment is a nucleic acid encoding a receptor

native to a human. The nucleic acids may be operably linked to a transcription promoter sequence and may further be incorporated into expression vectors suitable for production of recombinant FGF-R peptide.

5 Also included are isolated nucleic acids encoding a soluble human fibroblast growth factor receptor, preferably one homologous to h4 or h5. Protein products made by expressing such an isolated nucleic acid are provided.

10 A method is provided for making these proteins of newly recognized utility, e.g., fibroblast growth factor receptor activity, said method comprising expressing an isolated nucleic acid. Products produced by this method are now also available.

15 Additional methods are provided for making fibroblast growth factor receptor peptides by transforming a cell with a nucleic acid of at least about 21 bases of a sequence selected from the group consisting of:

- a) a DNA sequence in Figures 3, 4 or 9;
- b) a sequence encoding a polypeptide of Figures 3, 4 or 20 7; and
- c) a sequence substantially homologous to a sequence of Figures 3, 4 or 9.

25 Other methods for producing an antibody against a fibroblast growth factor receptor fragment are described, including a step of producing an antibody against a polypeptide epitope homologous to a sequence of at least six contiguous amino acids described in Figures 3, 4 or 7. The epitopes of most interest will be those from a signal segment, an IgI segment, an acidic segment, an IgII segment, an IgIII segment, 30 or an IgIIIT segment.

As a diagnostic use, these reagents provide a method for measuring a fibroblast growth factor or a fibroblast growth factor receptor in a target sample, said method comprising the steps of:

- 35 combining said target sample with a fibroblast growth factor receptor segment; and
- determining the extent of binding between said segment and said sample.

This invention also provides a transformed cell capable of expressing a polypeptide homologous to at least a portion of a human fibroblast growth factor receptor. A preferred embodiment is where the cell expresses a polypeptide
5 homologous to substantially the entire membrane bound or soluble form of a human fibroblast growth factor receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 compares the binding of various derivatives of FGF to FGF-R. Fig. 1(A) is a graph showing the percent binding inhibition of ^{125}I -labeled bFGF. Fig. 1(B) is an autoradiograph of bFGF cross-linked Swiss 3T3 cells subjected to gel electrophoresis.

Fig. 2(A) is an autoradiograph of cross-linked chicken membrane fractions and WGA eluates subjected to gel electrophoresis. Fig. 2(B) is a silver stained gel showing pure FGF receptor resulting from an affinity purification performed on the WGA-Sepharose 4B column chicken embryo eluate shown in Fig. 2(A).

Fig. 3 shows the nucleotide and amino acid sequence of a chicken bFGF receptor.

Fig. 4 shows the nucleotide and amino acid sequence of a human FGF receptor.

Fig. 5(A) represents an autoradiograph of a northern blot of chicken RNA probed with a full length cDNA chicken bFGF receptor under high stringency conditions. Fig. 5(B) represents an autoradiograph of a primer extension of chicken mRNA subjected to electrophoresis on an acrylamide sequencing gel.

Fig. 6 is a schematic of a chicken bFGF receptor indicating the (solid block) acidic domain; (cross-hatched block) transmembrane region; (flecked block) tyrosine kinase domain; (S), position of the SH cysteine residues (in contrast to the S designation of Table I); (W), position of tryptophan residue with respect to the first cysteine residue in the Ig-like domain.

Fig. 7 provides an amino acid sequence comparison of various different FGF receptor forms. The amino acid sequences of 4 human receptor forms are shown in comparison to a chicken FGF receptor sequence. Sequences which differ from the chicken FGF receptor sequence are outlined in open boxes. Transmembrane sequences are underlined. These DNA sequences are in GenBank/EMBL data bases under the following accession numbers: h2 is M34185, h3 is M34186, h4 is M34187, and h5 is M34188.

Fig. 8 provides a schematic representation of various different FGF receptors. The following structural features are identified: hydrophobic putative signal sequence (solid boxes), the highly acidic region (open boxes), transmembrane domain striped boxes), kinase 1 and kinase 2 domains (stippled boxes), and the divergent region of h4/h5 (zigzag line). Asterisks indicate the position at which h2 and h4 contain the sequence ArgMet, the chicken receptor contains a single Asn residue, and h3 and h5 contain no corresponding residues. Triangles indicate the position at which h3 contains a Glu residue and all other receptor forms contain a Lys residue. The numbers at the top of the figure indicate the degrees of amino acid identities between similar domains of the h2 human receptor and the chicken receptor.

Fig. 9 presents a comparison of various human FGF receptor genomic sequences with deduced amino acid sequences of FGF receptor cDNA clones. The sequence of a human genomic fragment obtained by PCR is shown in comparison to human and chicken cDNA sequences. A 1 kb intron separates genomic sequences encoding the Ig-like (Ig) domain and the highly acidic region. Dashed lines represent continuous sequence with no gaps. The deduced amino acid sequence shown for the chicken FGF receptor begins with the initiator methionine residue (1) and ends with the acidic region (EDDDDEDD; amino acids 125-132 in c1 FGF-R). The amino acid sequence shown for the human h2 FGF receptor begins with the initiator methionine residue (1) and ends with the acidic region (EDDDDDDD; amino acids 37-44 in h2).

Fig. 10 shows crosslinking of acidic or basic FGF to receptors in cells transfected with FGF receptor cDNAs. L6 cells (5×10^5) transfected with the cFGFR/pSV7d expression construct (lanes 1, 2, 7, and 8), the h2FGFR/pSV7d expression construct (lanes 3, 4, 9, and 10), or with vectors alone (lanes 5, 6, 11, and 12) were incubated with 0.1 pmoles of ^{125}I -aFGF (lanes 1-6) or ^{125}I -bFGF (lanes, 7-12) in the presence or absence of a 200-fold excess of unlabeled aFGF (lanes 2, 4, and 6) or bFGF (lanes 8, 10, and 12). Binding was performed for 30 minutes at 37°C. Cells were then washed twice with ice cold

DME H21 containing 20 mM HEPES pH 7.4, 0.2% gelatin, and twice with ice cold PBS. Disuccinimidyl suberate (DSS) was added to a final concentration of 0.15 mM and crosslinking was allowed to proceed for 15 minutes at 4°C. Samples were resuspended in sample buffer then subjected to SDS PAGE followed by autoradiography.

Fig. 11 illustrates acidic and basic FGF induction of a $^{45}\text{Ca}^{++}$ efflux from *Xenopus* oocytes injected with RNA encoding a chicken FGF receptor or the h2 human FGF receptor. The graphs show $^{45}\text{Ca}^{++}$ efflux from oocytes injected with chicken FGF receptor RNA (A and C, open squares), human h2 RNA (B and D, open squares), human h3 RNA (B and D, solid triangles) or water (A-D, solid squares). Injected oocytes were incubated with $^{45}\text{CaCl}_2$ for 3 hours at 19°C and then washed extensively. Groups of 5 oocytes were placed in individual wells of a 24 well plate and 0.5 ml of media was added. At 10 minute intervals, the media was removed for counting and fresh media was added. After 40 minutes, aFGF (panel A and B) or bFGF (panel C and D) were added to a final concentration of 0.5 nM. As a positive control, carbachol was added after 100 minutes. Each data point represents the average of triplicate wells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

OUTLINE

- I. General Description
 - A. FGF-R
 - 1. structural features
 - a. extracellular domain
 - i. signal sequence
 - ii. Ig domains
 - iii. acidic amino acid region
 - b. transmembrane segment
 - c. intracellular domain
 - i. tyrosine kinase
 - ii. insert
 - 2. function
 - a. bind FGF
 - b. bind to FGF-R peptide
 - c. tyrosine kinase activity
 - B. Physiological Functions
 - 1. cellular
 - 2. tissue differentiation
 - 3. organismal
- II. Polypeptides
 - A. Soluble Forms
 - B. Truncated Forms
 - C. Fusion Proteins
 - D. Genetic Variants (site-directed mutagenesis)
 - E. Compositions Comprising Proteins
- III. Nucleic Acids
 - A. Isolated Nucleic Acids
 - B. Recombinant Nucleic Acids
 - C. Compositions Comprising Nucleic Acids
- IV. Methods for Making FGF-R
 - A. Protein Purification
 - 1. affinity with derivatized FGF
 - 2. various ligands, same receptor
 - B. Expression of Nucleic Acids
- V. Antibodies
- VI. Methods for Use
 - A. Diagnostic
 - B. Therapeutic

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I. General Description

A first aspect of the invention provides homogeneous FGF-R peptides. These homogeneous FGF-Rs include a chicken basic fibroblast growth factor receptor and various human fibroblast growth factor receptors. Homogeneous polypeptides either having FGF-R ligand-binding activity or comprising a portion of the ligand-binding domain of an FGF-R are described. Notably, the present invention provides homogeneous

polypeptides corresponding to naturally occurring FGF-binding proteins having unexpected structural features. One class provides soluble proteins lacking a transmembrane segment, another class provides proteins possessing both a transmembrane segment and a tyrosine kinase domain. Both of these classes have an unexpected extracellular domain structure shorter than the corresponding chicken FGF-R. Experimental data indicating that a single receptor binds various FGF types is also described.

A second aspect of the invention provides isolated DNA sequences. These sequences encode polypeptides having FGF-R ligand-binding activity, including polypeptides which correspond to naturally occurring full-length fibroblast growth factor receptors. DNA sequences encoding a chicken bFGF-R or encoding various human FGF-Rs (hFGF-R) have been isolated. Also provided are cloning and expression vehicles containing the FGF-R encoding sequences. A DNA sequence encoding the full-length FGF receptor or an FGF-R polypeptide fragment can be operably linked to control sequences and expressed in a culture of a compatible transformed, transfected or infected host cells.

Methods of synthesizing growth factor receptor proteins and methods for providing analogues of the fibroblast growth factor receptors are provided.

The invention also provides antibodies to defined domains of the receptor. Still further aspects of the invention include methods for evaluating compositions which are agonistic or antagonistic to ligand and receptor interactions, particularly those which promote or inhibit binding interactions.

Diagnostic and therapeutic uses for the reagents provided herein are also described.

A. FGF Receptors

The fibroblast growth factor receptors (FGF-R) are receptors for the family of fibroblast growth factors (FGFs), as described above. See also P.L. Lee et al., Science 245:57-60, (1989), which is hereby incorporated herein by reference.

The FGF family consists of polypeptide growth factors characterized by amino acid sequence homology, heparin-binding avidity, the ability to promote angiogenesis, and mitogenic activity toward cells of epithelial, mesenchymal, and neural origin. The FGF family includes acidic FGF, basic FGF, the int-2 gene product, the hst gene product (Kaposi sarcoma-FGF), FGF-5, the keratinocyte growth factor, and FGF-6. Members of the FGF family appear to have roles in development, tissue repair, maintenance of neurons, and the pathogenesis of disease. Aberrant expression of FGFs may cause cell transformation by an autocrine mechanism. Moreover, FGFs may enhance tumor growth and invasiveness by stimulating blood vessel growth into the tumor or by inducing production of proteases such as plasminogen activator.

The term "ligand" refers to the molecules, usually members of the fibroblast growth factor family, that bind the domains involved in the growth factor binding. Also, a ligand is a molecule which serves either as the natural ligand to which the receptor binds, or a functional analogue which may serve as an agonist or antagonist.

As described herein, a chicken bFGF receptor is characterized by various identifiable structural features. The chicken and human FGF-R structures are generalized to define a structural nomenclature applicable to other FGF-Rs. General descriptions of protein structure and its relationship to nucleic acid sequences are discussed in J.D. Watson et al., Molecular Biology of the Gene, 4th Ed., vols. 1 and 2, Benjamin/Cummings, Menlo Park, California, (1987); and B. Alberts et al., Molecular Biology of the Cell, 2d Ed., Garland, New York, (1989), each of which is incorporated herein by reference. Common structural features of known FGF-Rs are described, including various naturally occurring soluble human FGF binding proteins. A human fibroblast growth factor receptor is a protein either derived from a natural human FGF-R gene, or which shares significant structural characteristics peculiar to a naturally occurring human receptor for FGF.

The isolated full-length chicken FGF-R mRNA contains a single hydrophobic segment similar to a membrane-spanning

segment (designated the transmembrane segment). The segments of FGF-R amino-proximal to the transmembrane segment are designated the extracellular domain, while the segments carboxy-proximal to the transmembrane segment are designated the intracellular domain. From the amino-terminus, the extracellular domain has an NH₂-terminal hydrophobic putative signal sequence, an immunoglobulin-like domain (designated IgI), and acidic segment, a second immunoglobulin-like domain (designated IgII), and a third immunoglobulin-like domain (designated IgIII). Although various structured features may be identified in the external domain of the FGF-R, the most important functional property which defines the domain is the binding to the receptor ligands, e.g., members of the FGF family. As discussed below, this function is correlated with the combined presence of IgII and IgIII domains.

The intracellular domain is characterized by the presence of a split tyrosine kinase structural domain and, in the chicken receptor, is about 424 residues long. Functionally, this domain is defined by its tyrosine kinase activity, typically modulated by ligand binding to the extracellular domain. A protein substantially lacks an intracellular domain when it lacks a prototypical intracellular domain, particularly lacking a tyrosine kinase domain.

Besides the chicken receptor, four unique human cDNA clones have been identified. These encode previously unknown FGF receptor variants which contain only two Ig-like domains. Two of the human clones encode membrane spanning receptors and two encode putative secreted forms. Both the forms exhibiting the 3 Ig-like or 2 Ig-like domain structures mediate biological responsiveness to acidic and basic FGF. Thus, the first Ig domain of the 3 Ig domain form may have a function other than binding of acidic and basic FGF. The multiple human receptor forms, are identical in some regions but are highly divergent in other selected regions of the extracellular domain. Two of the human variant receptors, h4 and h5, are likely to encode a secreted form of the FGF receptor.

A typical FGF-R nucleic acid sequence encodes a transitory NH₂-terminal hydrophobic sequence, which is usually

cleaved during the translocation process. The classical function of a signal sequence is to direct the nascent polypeptide chain to membrane bound ribosomes, thereby leading to membrane translocation. However, since the signal sequence is typically removed in the translocation process, the signal sequence is absent in a mature polypeptide.

The Ig-like domains (Ig domains) are characterized by three main features: (i) the presence of two characteristic cysteine residues in each domain; (ii) the presence of a consensus tryptophan residue 11 to 12 amino acids on the COOH-terminal side of the first cysteine residue in each Ig-like domain; and (iii) the presence of the consensus sequence, DXGXYXC, on the NH₂-terminal side of the second cysteine residue. The last feature is modified in the cases of the soluble receptor proteins, and substituted with an equivalently sized sequence.

Additional features characteristic of the Ig domains are apparent both in comparing the domains with one another, and comparing homologous domains of different receptor molecules. The amino-proximal Ig domain found in the chicken clone was designated IgI. As the chicken clone has three Ig domains, the domains have been numbered from the amino terminus. As indicated in Figure 6, the IgI domain includes the 45 amino acids flanked by a pair of cysteine residues. The chicken IgI domain has a high homology in sequence with the IgI domain found in the genomic sequence of the human FGF-R. However, the human forms appear to lack a domain corresponding to IgI.

The next Ig domain is designated IgII, and in the chicken receptor includes 51 amino acids between the two cysteine residues (see Figs. 3 and 6). As described below, this domain, in combination with the IgIII domain is involved with ligand binding. The polypeptide sequence homology of this domain between the chicken and human receptors is quite high, as shown by the sequence alignments in Fig. 7. It will be noted that the human receptors lack an Ig I domain but have IgII and IgIII domains. The cysteine residues used to delineate this domain are residues 176, 89, 87, 89, and 87 on

the amino proximal side, and 228, 141, 139, 141, and 139 on the carboxy proximal side for the chicken, h2, h3, h4 and h5 receptors, respectively.

5 The third Ig domain is designated IgIII and in the chicken receptor includes 63 amino acids between the two cysteine residues. See Figs. 3 and 6. Again, although the human receptors have only two domains, the domains correspond to IgII and IgIII. In both the chicken and human forms, the IgIII domain is that closest to the transmembrane segment. The
10 cysteine residues for the chicken, h2, h3, h4 and h5 receptors, respectively, used to delineate this domain are residues 274, 187, 185, 187, and 185 on the amino proximal side and residues 339, 252, 250, 253, and 251 on the carboxy proximal side.

The h4 and h5 soluble receptors have a substituted
15 terminal segment designated IgIIIT. This segment is a substituted terminal segment replacing part of the membrane bound to IgIII, and is 79 amino acids long. This sequence corresponds to amino acids 224 and 222 of h4 and h5, respectively, while preserving many of the features found in
20 the IgIII domain except of the DSGSYSC. It should be noted, however, the IgIIIT sequences are conserved between the soluble forms of the human FGF-R.

Between the first and second immunoglobulin-like domains, the FGF receptors (shown for the basic FGF-R, but the
25 same FGF-R binds both the acidic and basic FGFs) have a feature not found in other members of the immunoglobulin superfamily. There is a series of eight consecutive acidic residues (EDDDDEDD in the case of chicken, and EDDDDDDD in the case of human) followed by three serine residues and two additional
30 acidic residues (Figures 3 and 7). Although uninterrupted stretches of 7 to 35 acidic residues have been described for several intracellular proteins, in particular nuclear proteins, such acidic regions are unusual in the extracellular region of transmembrane receptor proteins.

35 The 5 receptor species (e.g. the chicken, h2, h3, h4 and h5 forms) also exhibit variability at a specific location between the conserved acidic region and the conserved second Ig-like domain (IgII). The h2 and h4 receptor forms contain

two amino acids (ArgMet) at positions 59 and 60, while the chicken receptor contains a single amino acid (Asn) at this position and the h3 and h5 receptor forms contain no corresponding amino acids at this position (see asterisks, Fig. 8).

Another unusual feature is the length of the juxtamembrane region, the region between the membrane spanning segment and the kinase domain. This region is normally conserved among receptor tyrosine kinases. For example, the juxtamembrane region is consistently 49 to 51 residues in length in the receptors for PDGF, CSF-1, epidermal growth factor (EGF), human epidermal growth factor-2 (HER2) and insulin. The FGF receptors with an intercellular domain have an unusually long juxtamembrane region of about 87 residues.

The cytoplasmic regions of the amino acid sequences are about 424 and 425 residues long, respectively for the chicken and human forms. These also contain a tyrosine kinase sequence (about residues 482 to 759, 395 to 672, and 393 to 670, respectively for the chicken, h2, and h3 forms). Overall, the kinase region of the bFGF receptors shares the most sequence identity (about 51 to 53%) with the PDGF and CSF-1 receptors. The bFGF receptors contain the GXGXXG motif and the conserved lysine residue (about residue 512) that form part of the adenosine 5'-triphosphate (ATP) binding site of tyrosine kinases. The bFGF receptors also contain the two characteristic tyrosine kinase motifs, HRDLAARNVL and DFGLAR, and a tyrosine (about residues 651, 564 and 562) at the position analogous to the major phosphorylation site of pp60^{v-src} (about Tyr 416).

The kinase coding sequence of the bFGF receptors, defined by homology to other tyrosine kinases, are split by an insertion of 14 amino acids. The length of the insertion in the kinase region is shorter than that found in the receptors for PDGF and CSF-1 (104 and 70 amino acids, respectively) and is similar to the length of the inserted sequence in the receptors for insulin and insulin-like growth factor-I.

The FGF-R appears to have three different biological functions. The first is the binding of ligands, usually the

FGF proteins or their analogues. These ligands or analogues may also serve as either agonists or antagonists. The ligand binding site is apparently in the extracellular domain. The receptor transduces a signal in response to ligand binding, and the result is a ligand modulated activity. As the likely ligand is a FGF, the signal will ordinarily be FGF-modulated.

A second biological activity relates to the tyrosine kinase enzymatic activity. This activity is typically activated in response to ligand binding. However, since the receptors are likely to function in a dimer state, the intrachain binding interactions may be considered another biological activity which may be mediated by blocking agents. this may serve as an additional means to modulate FGF-mediation of particular activities.

B. Physiological Implications

The interactions of FGFs with their receptors cause changes in, on particular cell types, cell morphology and cell transformation, cell proliferation, cell differentiation, cell senescence, heparin sensitivity, and heparin effects. The in vivo effects of FGF include, in particular organisms, modulation of various activities, e.g., limb regeneration, lens regeneration, angiogenic effects on both normal and tumor cells, wound healing, adipocyte differentiation, and growth of various neural and myoblast cells. FGFs also exhibit potent angiogenic activities. It is thought that the angiogenic activity of FGFs is due in large part to the chemotactic and mitogenic effects of these factors on endothelial cells. In addition, constitutive expression of FGFs has been shown to induce cellular transformation in transfected cells, indicating that autocrine or paracrine stimulation by FGFs may be involved in tumor formation. These diverse cellular and physiological effects foreshadow the central importance of these receptor-ligand interactions.

The compositions and cells comprising them can be used for diagnostic purposes and to study and treat diseases associated with FGF receptors. Cells expressing cloning vehicles containing defined sequences can be used to define specific sites of an FGF receptor necessary for effecting a

particular activity. Alternatively, these cells may be useful to assess the ability of a selected receptor to bind different ligands (FGFs and analogues) thereby providing a powerful tool for evaluating the potential of drugs for promoting or
5 inhibiting specific FGF-induced cellular responses.

Cells transfected, injected, infected or electroporated with DNA or mRNA containing a full length natural FGF-R sequence will often express the native or wild type receptor and respond accordingly. Specific concentrations
10 of a purified receptor or a receptor polypeptide fragment can be used to block the binding of the ligand (FGF) to native FGF receptors. Alternatively, antibodies to the receptor or fragment can have the same effect.

Homogeneous and defined polypeptides and DNA
15 sequences will find use in raising antibodies. In particular, antibodies against specific regions of the receptor, e.g., the ligand-binding domain, will find use in diagnostic testing. The reagents FGF-R, FGF-R polypeptides and antibodies to specific regions of the receptor can be used to study
20 regulation of FGF mediated activities. For example, FGF agonists should stimulate blood vessel development, an effect particularly beneficial in wound healing and in the growth of collateral blood vessels in ischemic areas of the heart. FGF antagonists should find use in preventing aberrant angiogenesis
25 as seen in diabetic retinopathy and rheumatoid arthritis or in controlling tumors by blocking proliferation of vascularization to a tumor.

II. Polypeptides

30 This invention includes fibroblast growth factor receptor polypeptides and proteins having FGF-R ligand-binding activity. The receptors of the present invention include FGF receptor amino acid sequences such as the amino acid sequences for a chicken bFGF-R and human FGF-R forms as shown in Figures
35 3, 4, and 7. Also included are homologous sequences, allelic variations, natural mutants, induced mutants, alternatively expressed variants, and proteins encoded by DNA which hybridize under high or low stringency conditions, to FGF receptor

encoding nucleic acids retrieved from naturally occurring material. Closely related FGF-receptors retrieved by antisera to FGF receptors are also included.

The symbols for the amino acid residues are shown in Table I.

Table I
Abbreviations for the Amino Acid Residues

A, Ala;	G, Gly;	M, Met;	S, Ser;
C, Cys;	H, His;	N, Asn;	T, Thr;
D, Asp;	I, Ile;	P, Pro;	V, Val;
E, Glu;	K, Lys;	Q, Gln;	W, Trp;
F, Phe;	L, Leu;	R, Arg;	Y, Tyr;

X, any amino acid and Z, termination.

Various new human FGF receptors have been cloned and characterized, as described further below. Of particular note, various shorter forms (h2 and h3) and soluble versions (h4 and h5) of FGF receptors have been discovered. The soluble proteins (e.g., forms lacking a transmembrane segment) which possess FGF binding capacity indicate that shorter forms will find therapeutic and/or diagnostic uses.

Typically, the fibroblast growth factor receptor peptides of the present invention will exhibit at least about 85% homology with the naturally-occurring receptors in the IgII and IgIII regions, usually at least about 90% homology, and preferably at least about 95% homology.

In particular, the ligand binding function is localized to the extracellular domain, and the soluble forms retain this particular function. Soluble fragments of FGF receptors should be useful in substituting for or interfering with the functions of the naturally soluble variants. Alternatively, the soluble forms may interfere with dimerization of FGF receptors, since the receptors may normally be in a dimer form. Receptor dimerization may be essential for proper physiological signal transduction.

The human receptors possessing a transmembrane segment are unusual in having only the IgII and IgIII of the three Ig domains. The absence of the IgI domain indicates that

certain functions may be absent in the human receptor, or, more likely, that the IgI domain is unnecessary in the human receptor. Data presented below shows that the IgI domain is not essential for ligand binding.

5 As used herein, the terms substantially pure and homogenous describe a protein which has been separated from components which naturally accompany it. Typically, a monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide backbone.
10 Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially pure protein will typically comprise over about 85 to 90% of a protein sample, more usually will comprise at least about 95%, and preferably will be over about 99% pure. Normally, purity is measured on a
15 polyacrylamide gel, with homogeneity determined by staining. For certain purposes high resolution will be used and HPLC or a similar means for purification utilized. For most purposes, a simple chromatography column or polyacrylamide gel will be used to determine purity.

20 A protein is substantially free of naturally-associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally
25 originates will be substantially free from its naturally-associated components. The term is used to describe receptors and nucleic acids which have been synthesized in heterologous mammalian cells or plant cells, *E. coli* and other prokaryotes.

30 A polypeptide is substantially an entire membrane bound form of an FGF-R when it is substantially a full length peptide corresponding to, or highly homologous to a naturally occurring membrane bound form of an FGF-R.

Whether soluble or membrane bound, the present invention provides for substantially pure preparations.
35 Various methods for their isolation from biological material may be devised, based in part upon the structural and functional descriptions contained herein.

FGF receptor peptides including chicken and human FGF

receptors may be purified using techniques of classical protein chemistry, see below. For example, a lectin affinity chromatography step may be used, followed by a highly specific ligand affinity chromatography procedure that utilizes an FGF conjugated to biotin through the cysteine residues of the FGF. Purified FGF-R receptors may also be obtained by a method such as FGF affinity chromatography using activated CH-Sepharose coupled to FGF through primary amino groups as described in Imamura, supra. This method, however, while resulting in a purified protein, may not provide a workable amount of purified protein (i.e. more than nanogram amounts).

Depending on the availability of specific antibodies, as provided herein, specific FGF receptors may also be purified using immunoaffinity chromatography. Antibodies prepared, as described below, may be immobilized to an inert substance to generate a highly specific affinity column. See Harlow and Lane, below.

By way of example and not limitation, one purification procedure may be used which takes advantage of the fact that labeled biotin-bFGF binds with high affinity to receptors in cells containing high amounts of those receptors. ¹²⁵I-labeled biotin-bFGF will bind to bFGF receptors in Swiss 3T3 cells and can be cross-linked to the receptor protein.

Various cell or tissue sources may be selected as starting materials usually selected due to an abundance of the desired receptor. Chicken embryos (day 6, stage 29-30) are preferred because they contain relatively large amounts of the receptor protein as determined by high-affinity binding of human and bovine bFGF. Embryo extracts can first be fractionated on wheat germ agglutinin (WGA) Sepharose 4B and the partially purified bFGF receptors then bound to biotin-bFGF. The receptor-ligand complex may be adsorbed to an avidin-agarose due to the high affinity interaction between the biotin and avidin moieties. The avidin-agarose columns may be eluted with compounds which dissociate the FGF from its receptor such as suramin or SDS. The chicken protein which bound to avidin-agarose in an FGF-dependent manner migrated at the expected size (130 kDa) of the bFGF receptor. See Fig. 2B.

To determine the amino acid sequence or to obtain polypeptide fragments of the receptor, the receptor may be digested with trypsin. Peptide fragments may be separated by reversed-phase high performance liquid chromatography (HPLC) and analyzed by gas-phase sequencing. Other sequencing methods known in the art may also be used.

The FGF receptors or the specific external regions of the receptors may be used to affinity purify respective FGFs. The external region comprising the ligand-binding domain of the chicken bFGF-R shown in Figure 3 extends from about amino acid 22 to about amino acid 374. The ligand-binding domain of the human FGF-R shown in Figure 4 extends from about amino acid 22 to about amino acid 285. The ligand-binding domain varies with different FGF receptors and may be anywhere from 5% to 100% of the extracellular region. The minimal amount of protein sequence necessary for ligand bonding may be determined by excising various segments of the extracellular domain and assaying ligand binding to the remaining sequence. Studies of ligand-receptor interaction indicate that at least the ligand-binding region is located in the extracellular region of the receptor is required. As used in this application, FGF receptor or FGF-R ligand-binding activity means having the ability to bind a fibroblast growth factor or other specific ligand. Usually these ligands will be members of the FGF family. Therefore the external region has utility in establishing FGF agonists or antagonists.

It is also likely that the FGF-R, like many other growth factor receptors, is found naturally in a multimeric protein complex, most likely in dimer form. Thus, other important regions of a receptor will be those, either extracellular or otherwise, which are involved in dimerization.

The intracellular regions of the receptors (e.g. starting at about amino acid 396 through the COOH-terminus for the chicken bFGF-R and about amino acid 307 through the COOH-terminus for the human FGF-R shown in Figures 3 and 4, respectively) may also be used as enzymes with tyrosine kinase activity. The bek gene has 84% amino acid sequence identity to the analogous region (tyrosine kinase region) of the chicken

bFGF-R. The flg has 99% homology with various sequences of the human FGF receptor described in Figure 4.

A signal or leader sequence directs a protein through the membrane of a cell. The signal sequences of the receptors may be used in conjunction with their respective receptors but may also be used with other proteins (e.g. amino acids about 1 through 21 of the N-terminal sequence comprise the leader or signal sequence of the chicken bFGF-R shown in Figure 3 and the human FGF-R shown in Figure 4).

The present invention also provides for analogues of the fibroblast growth factor receptor polypeptides. Such analogues include both modifications to a polypeptide backbone and variants and mutants of the polypeptides. Modifications include chemical derivatizations of polypeptides, such as acetylations, carboxylations and the like. They also include glycosylation modifications and processing variants of a typical polypeptide. These processing steps specifically include enzymatic modifications, such as ubiquitination. See, e.g., Hershko and Ciechanover (1982), "Mechanisms of Intracellular Protein Breakdown," Ann. Rev. Bioch., 51:335-364.

Other analogues include genetic variants, both natural and induced. Induced mutants may be derived from various techniques including both random mutagenesis of the encoding nucleic acids using irradiation or exposure to EMS, or may take the form of engineered changes by site-specific mutagenesis or other techniques of modern molecular biology. See, Sambrook, Fritsch and Maniatis (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press.

Besides substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides. Significant biological activities include ligand-binding, immunological activity and other biological activities characteristic of fibroblast growth factor receptor polypeptides. Immunological activities include both immunogenic function in a target immune system, as well as sharing of immunological epitopes for binding, serving as either a competitor or substitute antigen for a fibroblast

growth factor receptor epitope. As used herein, the term segment, as applied to a polypeptide, will ordinarily be at least about 5 contiguous amino acids, typically at least about 7 contiguous amino acids, more typically at least about 9 contiguous amino acids, usually at least about 11 contiguous amino acids, preferably at least about 13 contiguous amino acids, more preferably at least about 16 contiguous amino acids, and most preferably at least about 20 to 30 or more contiguous amino acids. Segments of a particular domain will be segments of the appropriate size within the corresponding domain.

For example, ligand-binding or other domains may be "swapped" between different new fusion polypeptides or fragments. Thus, new chimeric polypeptides exhibiting new combinations of specificities result from the functional linkage of ligand-binding specificities and intracellular domains. For example, the Ig domains may be substituted by Ig domains from other related polypeptides.

For immunological purposes, immunogens may be produced which tandemly repeat polypeptide segments, thereby producing highly antigenic proteins. Alternatively, such polypeptides will serve as highly efficient competitors for specific binding. Production of antibodies to fibroblast growth factor receptor polypeptides is described below.

The present invention also provides for other polypeptides comprising fragments of fibroblast growth factor receptors. Thus, fusion polypeptides between the receptors and other homologous or heterologous proteins are provided. Homologous polypeptides may be fusions between different growth factor receptors, resulting in, for instance, a hybrid protein exhibiting ligand specificity of one receptor and the intracellular domain of another, or a receptor which may have broadened or weakened specificity of binding. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a domain of a receptor, e.g., a ligand-binding domain, so that the presence or location

of a desired ligand may be easily determined. See, e.g., Dull et al., U.S. No. 4,859,609, which is hereby incorporated herein by reference. Other gene fusion partners include bacterial β -galactosidase, trpE Protein A, β -lactamase, alpha amylase, alcohol dehydrogenase and yeast alpha mating factor. See, e.g., Godowski et al. (1988), Science 241:812-816; and Experimental section below.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory, which are incorporated herein by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield, J. Amer. Chem. Soc. 85:2149-2156 (1963). The recombinant nucleic acid sequences used to produce fusion proteins of the present invention may be derived from natural or synthetic sequences. Many natural gene sequences are obtainable from various cDNA or from genomic libraries using appropriate probes. See, GenBank™, National Institutes of Health. Typical probes for fibroblast growth factor receptors may be selected from the sequences of Figures 3, 4, or 9 in accordance with standard procedures. Suitable synthetic DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts. 22:1859-1862 (1981). A double stranded fragment may then be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

III. Nucleic Acids

The present invention provides nucleic acid sequences encoding various FGF receptor sequences described above. Figures 3, 4, and 7 respectively set forth the corresponding cDNA sequences encoding chicken and human FGF receptors.

In Figure 3 showing the chicken bFGF-R, peptides sequenced from purified protein are underlined, including the

NH₂-proximal sequences from amino acids 35-53 (ala---arg), 56-67 (leu---arg), and 139-158 (glu---lys). The transmembrane sequence is indicated by a dark bar, a unique acidic amino acid region is outlined, cysteine residues are circled, potential N-linked glycosylation sites are indicated by a dot and the dashed underlining indicates the putative hydrophobic signal sequence. The amino acid sequence includes an in-frame stop codon (about residue -12) followed by an initiator methionine. The structural sequence begins at about amino acid 22.

In Figure 4 showing the human FGF-R, the methionine of codon ATG starting at about nucleotide 529 is the first amino acid of the FGF-R gene. For example, amino acid 22 of the receptor described in Figure 4 is an arginine residue (R) located two amino acids in from the left, two lines up from the bottom between "589" and "630" on page 1 of Figure 4.

Nucleic acids according to the present invention will possess a sequence which is either derived from a natural human, chicken, or other FGF-R gene or one having substantial homology with a natural FGF-R gene or a portion thereof.

Substantial homology in the nucleic acid context means either that the segments, or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, in at least about 80% of the residues, usually at least about 90%, more usually at least about 95%, preferably at least about 97%, and more preferably at least about 98 to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Figures 3, 4, or 9. Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of specificity. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14/25 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, Kanehisa, M. (1984), Nucleic Acids Res. 12:203-213, which is incorporated herein by reference.

Stringent hybridization conditions will typically include salt

concentrations of less than about 1 M, more usually less than about 500 mM and preferably less than about 200 mM.

Temperature conditions will typically be greater than 20°C, more usually greater than about 30°C and preferably in excess of about 37°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.

An isolated nucleic acid is one which has been substantially purified away from other sequences which normally accompany it, e.g., other cellular nucleic acid sequences. Usually, the term refers to a fragment of a genome which has been selectively cloned, isolated and purified to substantial homogeneity.

Probes may be prepared based on the sequence of the FGF receptor cDNAs provided in Figures 3, 4, and 9. The probes will include an isolated nucleic acid attached to a label or reporter molecule and may be used to isolate other FGF receptor nucleic acid sequences by standard methods. See, e.g. J. Sambrook et al., Molecular Cloning: A Laboratory Manual, vols. 1-3, CSH Press, N.Y. (1989), which is hereby incorporated herein by reference. Other similar nucleic acids may be selected for by using homologous nucleic acids. Alternatively, nucleic acids encoding these same or similar receptor polypeptides may be synthesized or selected by making use of the redundancy in the genetic code. Various codon substitutions may be introduced, e.g., silent changes thereby producing various restriction sites, or to optimize expression for a particular system. Mutations may be introduced to modify the properties of the receptors, perhaps to change the ligand binding affinities, the inter-chain affinities, or the polypeptide degradation or turnover rate.

The DNA compositions of this invention may be derived from genomic DNA or cDNA, prepared by synthesis or may be a hybrid of the various combinations. Recombinant nucleic acids comprising sequences otherwise not naturally occurring are also

provided by this invention. An isolated DNA sequence includes any sequence that has been obtained by primer or hybridization reactions or subjected to treatment with restriction enzymes or the like.

5 Synthetic oligonucleotides can be formulated by the triester method according to Matteucci, et al., J. Am. Chem. Soc., 103:3185 (1981) or by other methods such as commercial automated oligonucleotide synthesizers. Oligonucleotides can be labeled by excess polynucleotide kinase (e.g., about 10
10 units to 0.1 nmole substrate is used in connection with 50 mM Tris, pH 7.6, 5 mM dithiothreitol, 10 mM $MgCl_2$, 1-2 mM ATP, 1.7 pmoles ^{32}P -ATP (2.9 mCi/mmole) 0.1 mM spermidine, 0.1 mM EDTA). Probes may also be prepared by nick translation, Klenow fill-in reaction, or other methods known in the art.

15 cDNA or genomic libraries of various types may be screened. The choice of cDNA libraries normally corresponds to a tissue source which is abundant in mRNA for the desired receptors. Phage libraries are normally preferred, but plasmid libraries may also be used. For example, a keratinocyte cell
20 genomic or cDNA library would be preferred to isolate and clone a keratinocyte growth factor receptor. Embryonic or placental libraries can be used for int-2, FGF-5 and hst receptors and an endothelial cell library is preferred for acidic FGF receptors. Clones of a library are spread onto plates, transferred to a
25 substrate for screening, denatured and probed for the presence of desired sequences.

For example, with a plaque hybridization procedure, each plate containing bacteriophage plaques is replicated onto duplicate nitrocellulose filter papers (Millipore-HATF). The
30 phage DNA is denatured with a buffer such as 500 mM NaOH, 1.5 M NaCl for about 1 minute, and neutralized with, e.g., 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl (3 times for 10 minutes each). The filters are then washed. After drying, the filters are typically baked, e.g., for 2 hours at 80°C in a vacuum oven.
35 The duplicate filters are prehybridized at 42°C for 4-24 hours with 10 ml per filter of DNA hybridization buffer (20-50% formamide, 5X SSC, pH 7.0, 5X Denhardt's solution (polyvinylpyrrolidone, plus Ficoll and bovine serum albumin;

1X = 0.02% of each), 50 mM sodium phosphate buffer at pH 7.0, 0.2% SDS, and 50 µg/ml denatured salmon sperm DNA).

Hybridization with an appropriate probe may be performed at 42°C for 16 hrs with 10 ml/filter of 1×10^6 cpm/ml of DNA

5 hybridization buffer containing labeled probe. The final concentration of formamide is varied according to the length of the probe and the degree of stringency desired. See, e.g., J.G. Wetmur and Davidson, J. Mol. Biol. 31:349-370 (1968); and M. Kanehisa, Nuc. Acids Res. 12:203-213 (1984), each of which
10 is incorporated herein by reference, for a discussion of hybridization conditions and sequence homology.

An oligonucleotide probe based on the amino acid sequence of the two tryptic peptides of the purified chicken bFGF-R was used to screen a chicken embryo (day 6) cDNA library
15 under low stringency conditions. Sequences corresponding to TVALGSNVEFVCK and VYSDPQPHIQWLK, prepared using a commercial automated oligonucleotide synthesizer (Applied Biosystems) were used to obtain the chicken bFGF receptor clone described in Figure 3. This clone, or sequences derived from it, can be
20 used to isolate bFGF-Rs in other species as well as other FGF-Rs in a target species.

The probes described above which were used to isolate the chicken bFGF-R were also used to isolate a human bFGF receptor cDNA clone.

25 In accordance with this invention any isolated DNA sequence which encodes an FGF-R complete structural sequence can be used as a probe. Alternatively, any DNA sequence that encodes an FGF-R hydrophobic signal sequence and its translational start site may be used. Any isolated partial DNA
30 sequence which encodes an FGF-R activity (e.g. ligand-binding or FGF-R binding) is also part of this invention. Preferred probes are cDNA clones of each isolated FGF receptor.

The DNA sequences used in this invention will usually comprise at least about 5 codons (15 nucleotides), more usually
35 at least about 7 codons, typically at least about 10 codons, preferably at least about 15 codons, more preferably at least about 25 codons and most preferably at least about 35 codons. One or more introns may also be present. This number of

nucleotides is usually about the minimal length required for a successful probe that would hybridize specifically with an FGF receptor. For example, epitopes characteristic of an FGF-R may be encoded in short peptides. Usually the wild-type sequence will be employed, in some instances one or more mutations may be introduced, such as deletions, substitutions, insertions or inversions resulting in changes in the amino acid sequence to provide silent mutations, to modify a restriction site, or to provide specific mutations. The genomic sequence will usually not exceed about 200 kb, more usually not exceed about 100 kb, preferably not be greater than 0.5 kb.

Portions of the DNA sequence having at least about 15 nucleotides, usually at least about 15 nucleotides, and fewer than about 6 kd, usually fewer than about 1.0 kb, from a DNA sequence encoding an FGF receptor are preferred as probes. The probes may also be used to determine whether mRNA encoding a specific FGF-R is present in a cell or different tissues.

The natural or synthetic DNA fragments coding for a desired fibroblast growth factor receptor fragment will be incorporated into DNA constructs capable of introduction to and expression in an in vitro cell culture. Usually the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but may also be intended for introduction to, with and without and integration within the genome, cultured mammalian or plant or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA fragment encoding the desired receptor polypeptide, transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment and transcriptional and translational termination regulatory sequences operably linked to the polypeptide encoding segment. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known. See,

Sambrook et al. (1989). Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the fibroblast growth factor receptor DNA sequence may be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989); see also, Metzger et al. (1988), Nature 334:31-36.

Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferably, the enhancers or promoters will be those naturally associated with genes encoding the fibroblast growth factor receptors, although it will be understood that in many cases others will be equally or more appropriate. Other preferred expression control sequences are enhancers or promoters derived from viruses, such as SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Similarly, preferred promoters are those found naturally in immunoglobulin-producing cells (see, U.S. Patent No. 4,663,281, which is incorporated herein by reference), but SV40, polyoma virus, cytomegalovirus (human or murine) and the LTR from various retroviruses (such as murine leukemia virus, murine or Rous sarcoma virus and HIV) may be utilized, as well as promoters endogenous to FGF-R genes. See, Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, N.Y., 1983, which is incorporated herein by reference.

The vectors containing the DNA segments of interest (e.g., a fibroblast growth factor receptor gene or cDNA sequence or portions thereof) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for procaryotic cells, whereas calcium phosphate treatment may be used for other cellular hosts. See generally, Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press (1989), which is

incorporated herein by reference. The term "transformed cell" is meant to also include the progeny of a transformed cell.

As with the purified polypeptides, the nucleic acid segments associated with the ligand-binding segment, the extracellular domain and the intracellular domain are particularly useful. These gene segments will be used as probes for screening for new genes exhibiting similar biological activities, though the controlling elements of these genes may also be of importance.

IV. Methods for Making FGF Receptors

DNA sequences may also be used to express polypeptides which exhibit or inhibit FGF receptor activity. For example, a DNA sequence of from about 21 nucleotides (about 7 amino acids) to about 2.1 kb (about 700 amino acids) may be used to express a polypeptide having an FGF receptor specific activity, typically ligand-binding.

Large quantities of the receptor proteins may be prepared by expressing the whole receptor or parts of the receptor contained in the expression vehicles in compatible hosts such as E. coli, yeast, mammalian cells, insect cells or frog oocytes. The expression vehicles may be introduced into the cells using methods well known in the art such as calcium phosphate precipitation (discussed below), lipofection, electroporation or DEAE dextran.

Usually the mammalian cell hosts will be immortalized cell lines. To study the characteristics of an FGF-R and its corresponding growth factor, it will be useful to transfect, etc. mammalian cells which lack or have low levels of an FGF receptor where the signal sequence directs the peptide into the cell membrane. Cells without significant FGF receptors include lymphocytes, myocytes, green monkey cos-7 cells and Chinese hamster ovary cells (CHO). Transformed or transfected, etc., cells encode a receptor that is functionally equivalent to a wild-type receptor and confers a FGF-sensitive mitogenic response on the cell. Such cells will enable one to analyze the binding properties of various native FGFs. Transfected cells may also be used to evaluate a composition or drug's

effectiveness as an FGF antagonist or agonist. The level of receptor tyrosine kinase activity or the rate of nucleic acid synthesis can be determined by contacting transfected cells with drugs and comparing the effects of FGFs or their analogs on the drug-treated cells versus the controls. Although the most common prokaryote cells used as hosts are strains of E. coli, other prokaryotes such as Bacillus subtilis or Pseudomonas may also be used. The DNA sequence of the invention, including fragments or portions of the sequence encoding for an entire receptor, a portion of the receptor or a polypeptide having an FGF-R activity can be used to prepare an expression vehicle or construct for an FGF-R or polypeptide having an FGF-R activity. Usually the control sequence will be a eukaryotic promoter for expression in a mammalian cell. In some vehicles, the receptor's own control sequences may also be used. A common procaryotic plasmid vector for transforming E. coli is pBR322 or its derivatives (e.g. the plasmid pkt279 (Clontech)) (Bolavar et al., Gene, 2:95 (1977)). The procaryotic vectors may also contain procaryotic promoters for transcription initiation, optionally with an operator. Examples of most commonly used procaryotic promoters include the beta-lactamase (penicillinase) and lactose (lac) promoter (Cheng et al., Nature, 198:1056 (1977), the tryptophan promoter (trp) (Goeddell et al., Nucleic Acid Res., 8: 457 (1980)) the P_L promoter and the N-gene ribosome binding site (Shimatake et al., Nature, 292:128 (1981)).

Promoters used in conjunction with yeast can be promoters derived from the enolase gene (Holland et al., J. Biol. Chem., 256:1385 (1981)) or the promoter for the synthesis of glycolytic enzymes such as 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255 (1980)).

Appropriate non-native mammalian promoters might include the early and late promoters from SV40 (Fiers et al., Nature, 273:113 (1978) or promoters derived from murine molony leukemia virus, mouse mammary tumor virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct may be joined to an amplifiable gene (e.g. DHFR) so that multiple copies of the FGF receptor gene

may be made.

Prokaryotes may be transformed by various methods, including using CaCl_2 (Cohen, S.N., Proc. Natl. Acad. Sci. USA, 69:2110 (1972)) or the RbCl method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press 1982)). Yeast may be transformed using a method described by Van Solingen et al., J. Bacter., 130:946 (1977) and C.L. Hsiao et al., Proc. Natl. Acad. Sci. USA, 76:3829 (1979). With respect to eukaryotes, mammalian cells may be transfected using a calcium phosphate precipitation method described by (Graham and van der Eb, Virology, 52:546 (1978)), or by lipofectin (BRL) or retroviral infection (E. Gilboa, Experimental Manipulation of Gene Expression, Chap. 9, Academic Press P. 175 (1983)). The actual expression vectors containing appropriate sequences may be prepared according to standard techniques involving ligation and restriction enzymes (See e.g., Maniatis supra.) Commercially available restriction enzymes for cleaving specific sites of DNA may be obtained from New England BioLabs, Waltham, Massachusetts.

Clones are selected by using markers depending on the mode of the vector construction. The marker may be on the same or a different DNA molecule preferably the same DNA molecule. With mammalian cells the receptor gene itself may be the best marker. In procaryotic hosts the transformant may be selected by resistance to ampicillin, tetracycline or other antibiotics. Production of a particular product based on temperature sensitivity may also serve as an appropriate marker. Various methods may be used to harvest and purify the FGF-R receptor protein or peptide fragment. The peptide may be isolated from a lysate of the host. The peptide may be isolated from the cell supernatant if the peptide is secreted. The FGF-R peptide is then further purified as discussed above using HPLC, electrophoresis, affinity chromatography (preferably immuno-affinity or ligand affinity).

Another method which can be used to isolate cDNA clones of FGF-R related species involves the use of the polymerase chain reaction (PCR). (Saiki, R.K., et al. Science 230: 1350 (1985). In this approach two oligonucleotides

(27mers) corresponding to distinct regions of the FGF-R sequence are synthesized and then used in the PCR reaction to amplify receptor-related mRNA transcripts from an mRNA source. Annealing of the oligonucleotides and PCR reaction condition are performed under conditions of reduced stringency as described below in Example 2. The resulting amplified fragments are subcloned, and the resulting recombinant colonies are probed with ^{32}P -labeled full-length FGF-R cDNA using both high and low stringency conditions (see Examples 2 and 3). Clones which hybridize under low but not high stringency conditions represent FGF-R related mRNA transcripts. In addition this approach can be used to isolate variant FGF-R cDNA species which arise as a result of alternative splicing, see Frohman, M.A., et al., Proc. Natl. Acad. Sci. USA, 85: 8998 (1988).

V. Antibodies

Polyclonal and/or monoclonal antibodies to the various FGF receptors and peptide fragments may also be prepared. The term antibody is used both to refer to a homogeneous molecular entity, or a mixture such as a serum product made up of a plurality of different molecular entities. Peptide fragments may be prepared synthetically in a peptide synthesizer and coupled to a carrier molecule (i.e. keyhole limpet hemocyanin) and injected into rabbits over several months. The rabbit sera is tested for immunoreactivity to the FGF receptor protein or fragment. Monoclonal antibodies may be made by injecting mice with FGF-R protein, FGF-R polypeptides or mouse cells expressing high levels of the cloned FGF receptor on its cell surface. Monoclonal antibodies will be screened by ELISA and tested for specific immunoreactivity with the FGF receptor protein or polypeptides thereof. See, E. Harlow and D. Lane, Antibodies: A Laboratory Manual, CSH Laboratories (1988), which is hereby incorporated herein by reference. These antibodies will be useful in assays as well as pharmaceuticals.

Once a sufficient quantity of the desired fibroblast growth factor receptor polypeptide has been obtained, the protein may be used for various purposes. A typical use is the

production of antibodies specific for binding to these receptors. These antibodies may be either polyclonal or monoclonal and may be produced by in vitro or in vivo techniques.

5 For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit. The substantially purified antigen is presented to the immune system in a fashion determined by methods appropriate for the animal and other parameters well known to
10 immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally. Of course, another species may be substituted for a mouse or rabbit.

An immunological response is usually assayed with an
15 immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the antigen was produced. The immunoassay may be a radioimmunoassay, an enzyme-linked assay (ELISA), a fluorescent assay, or any of
20 many other choices, most of which are functionally equivalent but may exhibit advantages under specific conditions.

Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 to 10^{10} , or stronger will typically be made by standard procedures as described, e.g., in Harlow and Lane, Antibodies: A Laboratory Manual, CSH Laboratory (1988); or
25 Goding, Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York (1986), which are hereby incorporated herein by reference. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After
30 the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production
35 of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively

to selection of libraries of antibodies in phage or similar vectors. See, Huse et al., "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281 (1989), hereby incorporated
5 herein by reference. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and
10 conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Patents, teaching the use of
15 such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

20 VIII. Methods for Use

The present invention provides a fibroblast growth factor-receptor (FGF-R) purification method as well as a method for synthesizing FGF receptors within cells. Also provided are the homogeneous receptors produced by these methods, the
25 nucleic acid sequences encoding the receptors or portions of the receptors, as well as the expression vehicles containing these sequences, cells comprising the FGF-receptors and antibodies to the receptors. Of particular interest are the soluble forms of the receptors, which have binding sites which
30 may compete with receptors to bind FGF.

However, as indicated above, the FGF-R likely functions in a dimer state. The soluble forms of the receptor may interfere with the dimerization and may be effective in blocking signal transduction by a different mechanism from
35 competitive affinity for the FGF ligands. The soluble, or intracellular or transmembrane fragments of the various receptor forms are expected to interfere with dimer formation and thus can serve to block at least some types of, or some

fraction of signal transduction.

This observation provides a method for modifying in vivo a fibroblast growth factor receptor modulated activity comprising administering to a patient an amount of a fibroblast growth factor receptor blocking agent effective to inhibit fibroblast growth factor binding to fibroblast growth factor receptors. As discussed above, the FGF family of proteins have a significant role in regulating many important physiological processes. The soluble FGF-R polypeptides may be effective in modifying the extent of FGF modulation of these processes. For this reason, the soluble forms of the receptors may find use as competitive binding sites for FGF. Likewise, truncated FGF binding sites or binding sites which have been mutated, particularly those from the human forms described, may be equally effective in this effect at a lesser cost, both in terms of economics and in terms of medical side-effects upon administration.

The reagents provided herein will also find use in diagnosis of either FGF production or FGF-R production. Various medical conditions are indicated by an abnormal level of production of either of these proteins, including, e.g., Kaposi sarcoma, which produces Kaposi FGF, and diabetic retinopathy. Thus, diagnostic tests dependent upon these reagents now become available.

With the different FGF types, there is a likelihood that different types of receptors exist having variations in affinities for the various ligands. With the genes and proteins of the present invention, distinctions between various receptor types will be found. Thus, tissue markers should become available.

Since tumor growth is so dependent upon microvascularization, administration of the FGF-R may serve to prevent such and result in suppression of tumor growth. By prevention of the FGF activation, the present invention may be an important addition to the arsenal of agents for fighting tumor growth.

Viral infections may also be dependent upon binding to particular receptors for the invasion process. There is

suggestive evidence that HSV (Herpes simplex virus) infects by binding to FGF-R proteins. Thus, administration of therapeutically effective amounts of FGF-R soluble forms or fragments may serve as a prophylactic measure to minimize the risk of exposure to this, or other viruses, making use of this mechanism for cell entry. Again, the mechanism of protection may depend upon competitive binding, disruption of dimer structure, a combination, or another.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, one should titrate the dosage for treatment of particular conditions. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of dosages for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described in Gilman et al., Goodman and Gilman's: The Pharmacological Basis of Therapeutics, 7th Ed., MacMillan, New York (1985), which is hereby incorporated herein by reference. Because of the high affinity binding between FGF and its receptors, low dosages of these reagents would be initially expected to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than mM concentrations, typically less than about 10 μ M concentrations, usually less than about 100 nM concentrations, more usually less than about 1 nM, preferably less than about 10 pM (picomolar), more preferably less than about 100 fM (femtomolar), and most preferably less than about 1 fM, with an appropriate carrier.

The invention will better be understood by reference to the following illustrative examples.

35

Example 1

Characterization of a bFGF receptor.

¹²⁵I-labeled bFGF was first competitively bound to

Swiss 3T3 cells. As shown in Figure 1(A), ^{125}I -labeled bFGF (2 Ci/ μmol) was added to the confluent 3T3 cells (6 fmol of ^{125}I -labeled bFGF per 10^5 cells) in the presence of indicated concentrations of: unmodified bFGF (-X-); biotin-bFGF (solid square); the unbound fraction after biotin-bFGF was incubated with avidin-agarose, (open square); the unbound fraction after bFGF was incubated with avidin-agarose, (open triangle). Binding was performed for 30 min at 37°C in culture media (DME H21) containing 0.2% gelatin, and heparin (15 U/ml). The cells were washed three times with a buffer containing 20 mM HEPES (pH 7.4), 0.2% gelatin, and 150 mM NaCl. The radioactivity present was determined in a Beckman gamma counter. Maximal binding (0% inhibition) represents 5700 cpm of specific binding (nonspecific binding was 600 cpm). All determinations were made in triplicate. Recombinant human bFGF (Barr et al., *J. Biol. Chem.*, 263: 16471 (1988)) was iodinated using IODOBEADS (Pierce). The bFGF was iodinated using 0.5- 1mCi of ^{125}I per $1\text{ }\mu\text{g}$ FGF, 0.2M NaPi, pH 7.4, 2 IODOBEADS and incubated for 15 min. at room temperature, quenched with Na metabisulfite and excess KI. Iodinated bFGF was separated from unreacted free iodine by gel filtration on a PD 10 column equilibrated with 0.2M Na phosphate, pH 7.5, 0.2M NaCl, 0.2% gelatin. The bFGF was biotinylated using iodoacetyl-LC-biotin (Pierce) at a 4:1 molar excess of cysteine residues in 10 mM Tris-HCl (pH 8.0) for 5 hours at 4°C , according to the method of Yamamoto, et al., *FEBS Lett.* 176:75 (1984). Unreacted biotin was removed by gel filtration with PD 10 columns as described above (Pharmacia). During the purification procedure, modified bFGF was indistinguishable from unmodified bFGF in its ability to inhibit the binding of ^{125}I -labeled bFGF to high affinity bFGF receptors in Swiss 3T3 cells and in its ability to stimulate the phosphorylation of a 90 kD protein, known to be a substrate of bFGF-induced tyrosine kinase activity. See Fig. 1(A). The biotinylation reaction modified 90 to 95% of the bFGF molecules as measured by binding to avidin-conjugated agarose.

As shown in Fig. 1(B), cellular in situ bFGF receptors were cross-linked to labeled bFGF. ^{125}I -labeled biotin-bFGF or ^{125}I -labeled bFGF (0.1 pmol) was added to Swiss

3T3 cells (5×10^5 cells) in the presence or absence of unlabeled bFGF as indicated. The cells were washed and cross-linked with 0.15 mM disuccinimidyl suberate (DSS) (Pierce). The cells were then solubilized, subjected to SDS
5 polyacrylamide gel electrophoresis (PAGE) and ^{125}I -labeled proteins were detected by autoradiography. ^{125}I -labeled biotin-bFGF bound to bFGF receptors in Swiss 3T3 cells with high affinity (dissociation constant equals 1 nM) and was cross-linked to a 130 kD protein which comigrated with the bFGF
10 receptor cross-linked to ^{125}I -labeled bFGF.

Purified chicken bFGF receptor was prepared by homogenizing fresh day 6 chicken embryos (stage 29-30) with a Brinkmann polytron; (1500 embryos/batch); (1:1 v/v) in a final concentration of 0.25 M sucrose, 50 mM HEPES (pH 7.5), 2 mM
15 EDTA, 50 mM NaF, 150 μM sodium orthovanadate, 30 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (20 to 30 kallikrein international units (KIU)/ml, leupeptin (10 $\mu\text{g}/\text{ml}$), and pepstatin (1 $\mu\text{g}/\text{ml}$). The homogenate was centrifuged at 17,700g for 45 minutes at 4°C. The pellet
20 was resuspended in homogenization buffer (300 ml) and the resulting suspension was referred to as the membrane fraction (Mb). The membrane fraction was then incubated for 30 min at 4°C with an equal volume of 2X lysis buffer (1X lysis buffer consists of 10 mM Tris-HCl (pH 7.5)), 50 mM NaCl, 5 mM EDTA, 1%
25 Triton X-100, 50 mM NaF, 150 μM sodium orthovanadate, 30 mM sodium pyrophosphate, 1 mM PMSF, aprotinin (20 to 30 KIU/ml), leupeptin (10 $\mu\text{g}/\text{ml}$) and pepstatin (1 $\mu\text{g}/\text{ml}$), and then centrifuged at 31,000g for 30 min. The supernatant was applied
30 batchwise to a 150 ml WGA-Sepharose 4B column, washed with 300 ml of lysis buffer followed by 500 ml of column buffer which contained 20 mM HEPES (pH 7.5), 2 mM EDTA, 10% glycerol, 0.1% Triton X-100, 50 mM NaF, 150 μM sodium orthovanadate, 30 mM sodium pyrophosphate, 1 mM PMSF, aprotinin (20 to 30 KIU/ml), leupeptin (10 $\mu\text{g}/\text{ml}$) and pepstatin (1 $\mu\text{g}/\text{ml}$). The column was
35 eluted with column buffer containing 0.5 M N-acetylglucosamine. Peak protein containing fractions were combined and stored at -70°C.

To establish the presence of FGF-R in the embryo

membranes and WGA eluate, chicken bFGF receptor was cross-linked by incubating 10 μ l of the chicken embryo membrane fraction (Mb) or 100 μ l of the eluate from the WGA-Sepharose 4B column with 125 I-labeled bFGF (0.1 pmol) in the presence (+) or
5 absence (-) of a 200-fold excess of unlabeled bFGF for 30 min at 37°C (See Fig. 2(A)). DSS was added to a concentration of 0.15 mM, and the reaction mixture was incubated for 10 min on ice. Samples were subjected to SDS PAGE followed by
10 autoradiography. Specific binding and cross-linking of 125 I-bFGF to crude chicken embryo membrane fraction revealed only a single protein band of 150 kDa (Fig. 2(A)). After the molecular mass of bFGF was subtracted, the deduced size of the chicken bFGF receptor was 130-135 kDa.

As shown in Fig. 2(B), two large-scale ligand
15 affinity purifications were performed (each using the material from 20,000 embryos). The eluate from the WGA-Sepharose 4B column was incubated with biotin-bFGF prepared as described above (10:1 molar excess of ligand to receptor) and heparin at a concentration of 15 U/ml (to reduce low affinity binding) for
20 30 min at 4°C. The mixture was then cycled twice through a 10 ml avidin-agarose column (bFGF-agarose). To determine the nonspecific binding of protein to avidin-agarose (control), the eluate from the WGA-Sepharose 4B column was cycled through
25 avidin-agarose in the absence of biotin-bFGF (control). The columns were washed with 200 ml of column buffer used with the Sepharose column described above containing 0.2 M NaCl followed by column buffer without NaCl (300 ml) and then eluted with 10 mM suramin in column buffer. Four sequential 10 ml fractions
30 were collected (frac. 1-4) and samples of each fraction were subjected to SDS PAGE and stained with silver nitrate. As shown in Fig. 2(B), only a single protein bound to avidin-agarose in an FGF-dependent manner and it migrated at the expected size (130 kDa) of the bFGF receptor.

The eluted proteins were separated by acrylamide gel
35 electrophoresis and stained with Coomassie Blue. The band corresponding to the bFGF receptor was cut out and the protein electroeluted according to the method of M.W. Hunkapiller, et al., Meth. In Enzymol., 91: 227 (1983). This procedure

resulted in the purification of 2 to 5 ng of pure FGF receptor per chicken embryo with an overall recovery of 5%.

To further characterize the receptor, protein was digested with trypsin. Peptide fragments were isolated by reversed-phase high performance liquid chromatography (HPLC) and analyzed by gas-phase sequencing as described in Yarden et al., supra. From the two independent preparations, the amino acid sequences of 14 peptides, as shown in Fig. 3, were obtained. Three of the peptides were common to both preparations indicating identity between the two independent isolations. Four of the tryptic peptides (LILGKPLGEGCFGQVLA, IADFGLAR, MAPEALFDR and IYTHQSDVWSFGV, See Table I and Fig. 3) were homologous to consensus sequences for tyrosine kinase domains (Fig. 6). This was consistent with the finding that tyrosine kinase activity is associated with the bFGF receptor as described in Huang and Huang, J. Biol. Chem. 261:9568 (1986). Thus, the purified protein was determined to be a purified bFGF receptor in that it bound to bFGF, was the expected molecular weight of the receptor, and contained tyrosine kinase sequences.

As discussed above the amino acid sequences of 11 of the 14 peptides were identified in a previously published sequence of a partial human cDNA clone, termed flg (fms-like gene). See M. Ruta et al., Oncogene, 3: 9 (1988). That sequence was isolated on the basis of its homology to the proto-oncogene sequence and was not previously recognized to encode a transmembrane receptor protein.

Example 2

Isolation of a Full-Length Chicken bFGF Receptor cDNA Clone

A chicken embryo (day 6) cDNA library was constructed from size-selected poly A⁺ mRNA. 200 µg of poly A⁺ mRNA was size-fractionated on a 10%-30% sucrose gradient and fractions containing mRNA greater than or equal to 3.5 Kb were pooled. 5 µg of the sized mRNA was used to generate the cDNA according to the method of U. Gubler and B. Hoffman, Gene 25:263 (1983) using a cDNA synthesis kit from Pharmacia (cat.#27-9260-01). The synthesized cDNAs were size-selected for cDNAs greater than

or equal to 2.0 kb, and the sized cDNAs were then cloned into the Eco RI site of the bacteriophage vector ZapII (Stratagene, cat.#236211). The resultant cDNA library contained 2.0×10^6 independent recombinants.

5 The library was screened with a ^{32}P -labeled
oligonucleotide probe that encoded the two contiguous peptides
shown in Fig. 3 (TVALGSNVEFVCK and VYSDPQPHIQWLK). The
oligonucleotides were prepared using a commercial automated
oligonucleotide synthesizer. Two 43-45 base oligonucleotides
10 containing a 12 base overlapping complementary sequence were
annealed and labeled by Klenow fill-in with dNTP's (-dCTP),
 ^{32}P -dCTP, and DNA polymerase Klenow fragment yielding a 70 bp
labeled probe. Filters were hybridized under low stringency
conditions (20% formamide, 5X standard saline citrate (SSC) and
15 5X Denhardt's solution at 42°C) and washed with 0.2X SSC at
42°C. Twenty-five positive clones were isolated following 3
rounds of plaque purification. Of the 25 positive clones, 11
hybridized at high stringency to the human FGF-R cDNA labeled
by nick translation and used as a probe (see Example 3). All
20 of the 11 clones were essentially identical except for
variation in length at the 5' end of the clones. The amino
acid sequence of the largest clone (3.2 kb) contained the
sequence of all 14 of the receptor peptides obtained in the
protein purification described above (See Fig. 3) and contained
25 the complete coding sequence of the FGF-R. The transmembrane
region and the hydrophobic signal sequence were identified by
Kyte and Doolittle hydropathy analysis as described in Kyte and
Doolittle, J. Mol. Biol., 157:105 (1982).

A single hybridizing band of approximately 3.5 Kb was
30 identified by probing chicken embryo poly(A)⁺ RNA (5 µg) with
full-length chicken bFGF receptor cDNA under high stringency
conditions (50% formamide), 5X Denhardt's solution and 5X SSC
at 42°C. Filters were then washed with 0.2X SSC at 65°. The
3.5 kb single hybridizing band identified by the RNA blot
35 analysis is shown in Fig. 5(A). Primer extension experiments
with an oligonucleotide complementary to a sequence near the 5'
end of the clone were performed. Chicken embryo poly(A)⁺ RNA
(5 µg) was denatured with 10 mM methylmercury, annealed to

³²P-labeled primer (5' CTGCACGTCATCGCGCA-3') and extended with murine Moloney leukemia virus reverse transcriptase. (See Figure 5(B): lane (S) represents ³²P-labeled DNA molecular size standards (1 kb); Lane (E) represents extended fragment (523 nucleotides); Lanes (G, A, T, and C) represent a 5% acrylamide sequencing gel. The data predicted that the mRNA of the receptor was 48 nucleotides longer than the isolated clone.

The amino acid sequence of the longest open reading frame (2.4 kb) included an in-frame stop codon (amino acid residue -12) followed by an initiator methionine (residue 1) and the entire receptor coding sequence (Fig. 3). The cDNA encoded a protein with a deduced molecular mass of 91.7 kD that had features found in several known growth factor receptors. It contained a single-membrane spanning region, an NH₂-terminal hydrophobic signal sequence, three extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (Fig. 6). Eleven potential N-linked glycosylation sites were also found. N- and O-linked glycosylation of the chicken bFGF receptor may account for the disparity between the observed size of the bFGF receptor and the size predicted from the cDNA sequence.

Three immunoglobulin-like domains in the putative extracellular region were identified on the basis of three criteria: (i) the presence of two characteristic cysteine residues in each domain; (ii) the presence of a consensus tryptophan residue 11 to 12 amino acids on the COOH-terminal side of the first cysteine residue in each immunoglobulin-like domain; and (iii) the presence of the consensus sequence, DXGXYXC, on the NH₂-terminal side of the second cysteine residue in each immunoglobulin-like domain. The interleukin-1 (IL-1) receptor also has three immunoglobulin-like domains, and bFGF has 25-30% sequence identity to IL-1. Five immunoglobulin-like domains are present in the receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1).

Between the first and second immunoglobulin-like domains, the bFGF receptor has a feature not found in other members of the immunoglobulin superfamily. There is a series

of eight consecutive acidic residues (EDDDDEDD) followed by three serine residues and two additional acidic residues (Fig. 3). Although uninterrupted stretches of 7 to 35 acidic residues have been described for several intracellular proteins, in particular nuclear proteins, such acidic regions are unusual in the extracellular region of transmembrane receptor proteins.

Another unusual feature is the length of the juxtamembrane region, the region between the membrane spanning segment and the kinase domain. This region is normally conserved among receptor tyrosine kinases. For example, the juxtamembrane region is consistently 49 to 51 residues in length in the receptors for PDGF, CSF-1, epidermal growth factor (EGF), human epidermal growth factor-2 (HER2) and insulin. The bFGF receptor has an unusually long juxtamembrane region of about 87 residues.

The cytoplasmic region of the amino acid sequence is about 424 residues long and contains a tyrosine kinase sequence (about residues 482 to 759). Overall, the kinase region of the bFGF receptor shares the most sequence identity (about 51 to 53%) with the PDGF and CSF-1 receptors. The bFGF receptor contains the GXGXXG motif and the conserved lysine residue (about residue 512) that form part of the adenosine 5'-triphosphate (ATP) binding site of tyrosine kinases. The bFGF receptor also contains the two characteristic tyrosine kinase motifs, HRDLAARNVL and DFGLAR, and a tyrosine (about residue 651) at the position analogous to the major phosphorylation site of pp60^{v-src} (about Tyr 416).

The kinase coding sequence of the bFGF receptor, defined by homology to other tyrosine kinases, is split by an insertion of 14 amino acids. The length of the insertion in the kinase region is shorter than that found in the receptors for PDGF and CSF-1 (104 and 70 amino acids, respectively) and is similar to the length of the inserted sequence in the receptors for insulin and insulin-like growth factor-I.

Example 3

Full Length Human FGF Receptor cDNA Clone Preparation

A human FGF receptor cDNA clone was isolated from a human endothelial cell cDNA library obtained from E. Sadler (R.D. Ye T-C Wun & J.E. Sadler, J. Biol. Chem., 262: 3718-3725 (1987)) using the same oligonucleotide probe described in Example 2.

The endothelial library was hybridized at high stringency with labeled probe 1×10^6 cpm/ml (50% formamide, 5X SSC, 5X Denhardt's, 10mM NaPO₄, pH 6.5, 100 µg/ml salmon sperm DNA at 42°C, (16-24 hrs) and washed at 65°C with 0.2X SSC, 0.1% SDS.

From the initial screening of the human endothelial cell cDNA library, four clones were identified and purified through 3 rounds of plaque purification. The cDNA inserts from three of these clones generated identical sequences and contained sequences highly homologous to the sequences of tryptic fragments from the purified chicken bFGF-R. The amino acid and nucleic acid sequence of the largest clone (approximately 3.6 kb) is set forth in Figure 4. Amino acids about 1-21 represent the hydrophobic signal sequence, about 22-285 the extracellular region containing the ligand-binding domain, about 286-306 the transmembrane region and about 307-731 the cytoplasmic region containing tyrosine kinase domain. This method also isolated other highly related human FGF receptors.

Example 4

Human aFGF-R cDNA Clone Preparation

Human endothelial cell or placental libraries are screened with full-length FGF-R probes or probes containing a portion of the sequence for FGF-R. Hybridization is performed at low stringency conditions and washed in increments of increasingly higher stringency. The low and high stringency conditions described in Examples 2 and 3 are followed. Between each increment, autoradiography is performed. Clones which are positive through to the most stringent conditions are most related to the bFGF receptors previously described in Examples 2 and 3. Clones which are positive at relaxed stringency but are no longer positive at high stringency conditions are more

distantly related. All related but not identical (to Figure 4) clones are determined by restriction mapping and DNA sequencing. All related clones are selected, subcloned and expressed. The expressed FGF-related cDNAs are then tested for
5 their ability to bind the various FGFs, i.e. acidic FGF.

Alternatively, two probes are designed, one probe containing intracellular FGF-R sequence and the other extracellular FGF-R sequence. Triplicate filters are made. One filter is hybridized at high stringency (see Examples 2 and
10 3) with the intracellular FGF-R probe. Two filters are hybridized with the extracellular probe, one filter at high stringency and one at low stringency. Since acidic and basic FGFs have only 55% sequence identity, their receptors may also exhibit about 55% sequence identity in the ligand-binding
15 domain. Clones which are positive at high stringency to the intracellular probe and positive only at low stringency to the extracellular probe are FGF-R related receptors. Thus clones are selected, restriction mapping performed, sequenced, and expressed. The expressed receptors are tested for their
20 ability to bind to various FGFs, e.g., acidic FGF.

Example 5

Characterization of human FGF-R cDNA clones

Plasmid Constructions.

25 For transfection experiments, full-length chicken FGF receptor cDNA containing 46 nucleotides of 5' nontranslated sequence and the entire 3' nontranslated sequence, and full-length human h2 cDNA containing 13 nucleotides of 5' nontranslated sequence and the entire 3' nontranslated sequence
30 were individually subcloned into the BamHI/SalI sites of the mammalian expression vector pSV7d (P. Luciw, Chiron Corporation). This placed the receptor cDNA fragments in the proper orientation directly downstream from an SV40 promoter element.

35 To prepare constructs to be used as templates for generating in vitro transcribed RNAs, full-length chicken FGF receptor cDNA was subcloned into the BamHI/SalI sites of Bluescript Sk (Stratagene) and full-length human FGF receptor

cDNAs (h2 and h3) were subcloned into the PstI/SalI sites of Bluescript KS. This placed the receptor sequences directly downstream from the T7 RNA polymerase promoter element. To enhance the possibility of efficient translation, ATG sequences upstream of the initiator methionine residue were removed prior to subcloning, leaving 46 and 13 nucleotides of intact 5' nontranslated sequence for the chicken and human constructs, respectively.

Cell Lines and Transfections.

Rat L6 skeletal muscle myoblasts (ATCC CRL 1458) were grown in DME H21 containing 10% fetal calf serum and transferred into Opti-MEM (GIBCO) just prior to transfection. Within 24 hours after plating, 1×10^6 cells were cotransfected with 20 μ g of the appropriate expression construct (either cFGFR/pSV7d or h2FGFR/pSV7d) and 1 μ g of a vector containing the neomycin resistance gene (pSV2neo). Cells were transfected using 50 μ g of Lipofectin (Bethesda Research Laboratories) following the protocol provided by the manufacturer. Sixteen hours later, an equal volume of DME H21 media containing 20% fetal calf serum was added. After 48 hours, cells were harvested and passaged (1:10) into selection media (DME H21, 10% fetal calf serum, 500 μ g/ml geneticin (GIBCO). Transfectant colonies were assayed for expression of the FGF receptor by immunoblotting with anti-receptor peptide polyclonal antisera.

Affinity Labeling.

Recombinant human aFGF and human bFGF were generously donated by Chiron Corporation and indicated. For affinity labeling experiments, 5×10^6 cells were incubated for 30 minutes at 37°C with 0.1 pmoles of 125 I-aFGF or 125 I-bFGF in the presence or absence of a 200-fold excess of the corresponding unlabeled ligand. The cells were then washed once with ice cold DME H21 containing 20 mM HEPES pH 7.4, 0.2% gelatin, and twice with ice cold PBS. Disuccinimidyl suberate was added to a final concentration of 0.15 mM and incubations were allowed to proceed for 15 minutes at 4°C. The crosslinking agent was then removed and the cells were resuspended in sample buffer containing 100 mM dithiothreitol,

boiled for 5 minutes, and subjected to SDS PAGE followed by autoradiography.

In vitro Transcription of RNA.

Prior to transcription, plasmid constructs were linearized with XhoI. RNAs were transcribed from the linearized templates using T7 RNA polymerase in the presence of 500 μ M rNTPs (200 μ M rGTP) and 500 μ M 5'GpppG^{3'}, (Pharmacia). Following incubation at 4°C for 2 hours, transcription reactions were treated with RNase-free DNase, phenol extracted, ethanol precipitated, dried and resuspended in water.

Injection of Oocytes.

Animals were anesthetized in a solution of 0.06 percent ethyl p-aminobenzoate. Oocytes were surgically removed and manually dissected into clusters containing 10-20 oocytes. Clusters were incubated in modified Barth Saline (See Maniatis, et al., Molecular Cloning: A Laboratory Manual, CSH Press (1982), which is incorporated herein by reference.) MBSH containing 1 mg/ml Type II collagenase (Sigma) for 2 hours at room temperature and then washed extensively with MBSH containing 2 mg/ml bovine serum albumin (BSA). Individual oocytes were maintained at 19°C in MBSH (1 mg/ml BSA).

Oocytes were injected into the vegetal pole with 50 nl of water or RNA solution (1 μ g/ μ l in water). Following injection, oocytes were incubated at 19°C for 48 hours before performing ⁴⁵Ca⁺⁺ efflux assays.

⁴⁵Ca⁺⁺ Efflux Assays.

Groups of 50 injected oocytes were added to single wells of a 24 well plate and washed four times with 0.5 ml of a Ca⁺⁺-free MBSH solution containing no BSA. Oocytes were then incubated in 0.5 ml of the wash solution containing ⁴⁵CaCl₂ (100 μ Ci/ml) for 3 hours at 19°C. Following incubation, oocytes were washed six times with 0.5 ml of MBSH (1 mg/ml BSA), then transferred to another 24 well plate (5 oocytes per well). All subsequent washes and incubations were performed using 0.5 ml of MBSH containing 1 mg/ml BSA. At 10 minute time intervals, conditioned supernatants were removed from each well and replaced with fresh media. The conditioned media samples were counted individually in a Beckman scintillation counter.

When background efflux stabilized, ligands were added to the specified concentrations and media collections were continued.

In 2 out of 16 experiments, oocytes injected with water and stimulated with either aFGF or bFGF exhibited $^{45}\text{Ca}^{++}$ efflux levels similar to those obtained from oocytes injected with FGF receptor RNA. We have not determined the reason for these unexpected responses, but it is possible that they were due to expression of endogenous FGF receptors on contaminating follicular cells, or on the surface of the oocytes themselves. In all other experiments the water injected oocytes had no significant efflux response whereas the receptor RNA response to FGF was ten to forty fold over the basal measurement.

Receptor levels in injected oocytes have not been measured because our anti-receptor polyclonal antiserum nonspecifically recognizes an abundant oocyte protein of approximately the same molecular weight as the FGF receptor on western blots. Furthermore, the levels of exogenous receptors expressed in oocytes appears to be quite low.

Isolation and Characterization of Human cDNA Clones.

Complementary DNA libraries from human placenta and human umbilical vein endothelial cells were generously donated by J. Evan Sadler (Washington University School of Medicine, St. Louis). The libraries were screened with ^{32}P -labeled oligomers identical to those previously used to identify chicken FGF receptor cDNA clones. Filters were hybridized and washed under high stringency conditions using standard methods. A total of 7 positive clones were isolated after screening 250,000 phage from both libraries. The 4 clones described in this report (h2, h3, h4, and h5) were sequenced by the dideoxy chain termination method, using the Sequenase system (United States Biochemical Corporation). Clones h2, h3, and h4 were obtained from the endothelial cell library and clone h5 was obtained from the placenta library. Nucleotide sequence analyses revealed that all four clones contained identical 5' nontranslated sequences and had poly-A tracts at their 3' ends. However, only the poly-A tract at the 3' end of h2 was preceded upstream by a consensus poly adenylation signal sequence (AATAAA; 37), indicating that internal priming was responsible

for the poly A tracts at the 3' ends of the other clones. The h2, h3, h4, and h5 cDNAs contained 0.93 kb, 0.78 kb, 0.95 kb, and 0.2 kb of 3' nontranslated sequence, respectively. The 3' nontranslated sequences of h2 and h3 were identical and the 3' nontranslated sequences of h4 and h5 were also identical. In contrast, the h2/h3 3' nontranslated sequences were entirely different from the 3' nontranslated sequences of h4/h5.

Polymerase Chain Reactions.

Amplification reactions (42) were carried out using one primer corresponding to the human highly acidic region (approximately amino acids 44-52 in h2; S'GTTTCTTTCTCCTCTGAAGAGGAGT-3') and one degenerate primer corresponding to the IgI domain of the chicken FGF receptor (approximately amino acids 58-69; 5'- GA(TC)GACGTGCAG (A/T) (G/C)CATCAACTGGGTGCGTGATGG-3'). In additional reactions, we used the primer from the human highly acidic region and a second primer derived from the 5' nontranslated region of the human FGF receptor (5'-GAGGATCGAGCTCACTGTGGAGTA-3'). Reaction mixtures contained 750 ng of human genomic DNA, 10 pmoles of each primer, 200 μ M of each of the four dNTPs, and 1 unit of Taq polymerase (Perkin Elmer Cetus) in 50 μ l of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 ng/ml BSA. Reactions were carried out in an Ericomp twin block system. Thirty one cycles were performed, consisting of denaturation at 94°C for 50 seconds, annealing at 65°C for 1 minute, and extension at 72°C for 3 minutes.

Isolation and Characterization of Four Unique Human FGF Receptor cDNAs.

The chicken basic FGF receptor contains a single transmembrane domain, an extracellular region containing 3 Ig-like domains and a highly acidic domain, and an intracellular region containing a split tyrosine kinase domain. The chicken FGF receptor cDNA is highly homologous to a previously published partial cDNA (hflg) which encodes a tyrosine kinase that, at the time of its description was of unknown function. The high degree of identity (95 percent) between the chicken bFGF receptor and human flg suggested that hflg was the human counterpart of the bFGF receptor. To obtain full-length human

FGF receptor cDNAs, oligonucleotide probe based on the hflg cDNA sequence was used to screen a human umbilical vein endothelial cell cDNA library and a human placenta cDNA library. From the initial screenings of 250,000 plaques from each library, four positive clones were isolated from the endothelial cell library and three from the placenta library.

The cDNA clones could be divided into two classes based on different patterns of restriction maps at their 3' ends. One of these classes derived from cDNA clones which were much shorter in length. Representatives of each class were present in the clones isolated from either library. Two clones (h2 and h3) representing the class of larger cDNA clones, and two clones (h4 and h5) representing the class of shorter cDNA clones were sequenced in their entirety. The deduced amino acid sequences of the four human receptor forms are shown in comparison to the chicken FGF receptor sequence in Figure 7. A schematic representation of the different receptor forms is shown in Figure 8.

The predicted amino acid sequences of the h2 and h3 clones are virtually identical and differ only by three amino acids (amino acids 59, 60, and 103 in h2, Fig. 7). At the nucleotide level, h2 and h3 differ only at the positions encoding these three amino acid residues. The h2/h3 open reading frames include a hydrophobic signal sequence and the unusual acidic domain (8 consecutive acidic residues with accompanying residues) that was initially noted in the published sequence of the chicken FGF receptor cDNA. The extracellular domains of h2 and h3 are highly homologous to the chicken FGF receptor except that h2 and h3 lack the sequences of one Ig-like domain (labeled I in Fig. 8). The transmembrane region and cytoplasmic domains are highly homologous to the corresponding domains of the chicken FGF receptor.

The coding sequences of the short cDNA clones, h4 and h5, differ only by two amino acids (positions 59 and 60 in h4; the nucleotide sequences of h4 and h5 differ only at the positions encoding these two residues). The signal sequence, acidic region and one of the Ig-like domains (IgII) are essentially identical to the corresponding regions of h2 and

h3. The distinctive feature of h4 and h5 is the Ig-like domain (IgIII) nearest the transmembrane domain. Approximately half of this domain is identical to the corresponding sequence of h2 and h3. However, the carboxyl terminal half of this Ig-like domain is unrelated to h2 and h3 sequences. Unlike h2, h3, and the chicken FGF receptor cDNA, h4 and h5 do not encode a hydrophobic membrane spanning region or a cytoplasmic domain.

The sequences of all of the human cDNAs which have been isolated contain only 2 Ig-like domains. To determine whether the human FGF receptor gene contains sequences encoding the first Ig-like domain (IgI), polymerase chain reactions were performed on genomic DNA isolated from human foreskin fibroblasts (HFFs). For these experiments, we utilized one amplifying primer based on the sequence of the IgI domain of the chicken receptor (corresponding to amino acids 58-69), and a second primer based on sequence from the acidic region of the human receptor (amino acids 44-52 in h2). Using these primers, a single 1.3 kb genomic fragment was amplified. As shown in Figure 9, this fragment contained coding sequences homologous (approximately 83 percent amino acid identity) to the IgI domain of the chicken FGF receptor. In addition, an intron sequence of approximately 1.0 kb separates these coding sequences from sequences encoding the highly acidic region of the receptor. Thus, the human FGF receptor gene clearly contains sequences encoding the IgI domain not found in the human cDNA clones. Furthermore, the presence of an intron between the IgI domain sequence and the acidic region sequence suggests that expression of 2 or 3 Ig domain forms may be regulated by alternative splicing.

To determine whether a 3 Ig domain form of the receptor is expressed in HFF cells, we performed PCR on cDNA generated from HFF mRNA. Using the primers described above, a single 0.24 kb fragment was amplified from HFF cDNA. This fragment contained sequences encoding the IgI domain and the acidic region, but no intron sequences. Thus, we conclude that HFF cells transcribe a 3 Ig domain form of the receptor. To determine whether HFF cells also express a 2 Ig domain form of the receptor, we utilized the acidic region primer and a second

primer based on sequence from the 5' nontranslated region of the human FGF receptor. In these reactions a 0.23 kb fragment was amplified which, in the same manner as our cDNA clones, was missing sequences corresponding to the IgI domain. Thus, a 2
5 Ig domain form of the receptor is also transcribed in HFF cells.

Receptors Containing 3 Ig-like and 2 Ig-like Domains Bind Acidic FGF and Basic FGF.

Since the 3 Ig domain receptor (initially isolated
10 from a chicken cDNA library) was purified on the basis of its affinity for basic FGF, it was of interest to determine whether this receptor also binds acidic FGF. To address this question, the 3 Ig domain chicken receptor was expressed in rat L6 myoblasts, a cell line which normally does not express FGF
15 receptors. In addition, the 2 Ig domain human h2 receptor was also expressed in L6 cells. Figure 10 shows an affinity labeling experiment performed with transfected cells. Cells were incubated with either ^{125}I -aFGF or ^{125}I -bFGF and bound ligand was crosslinked in the presence of disuccinimidyl
20 suberate (0.15 mM). Using either ligand, single crosslinked bands were seen in cells transfected with receptor cDNAs (lanes 1, 3, 7, and 9), but not in cells transfected with vector alone (lanes 5, 6, 11, and 12). Subtraction of the molecular weight of FGF (17 kd) from the size of the crosslinked complexes
25 yields estimated molecular weights of 145 kd for the 3 Ig domain form of the receptor and 125 kd for the 2 Ig domain form of the receptor. Excess unlabeled ligands block formation of the crosslinked complexes (lanes 2, 4, 8, and 10). These results demonstrate that both the 3 Ig domain form and the 2 Ig
30 domain form of the FGF receptor are capable of binding either acidic or basic FGF. Scatchard binding analyses indicate that half-maximal binding of ^{125}I -aFGF to either the 3 Ig domain form or the 2 Ig domain form occurs at a concentration of 0.05 nM. Similarly, half-maximal binding of ^{125}I -bFGF to either the
35 3 Ig domain form or the 2 Ig domain form occurs at 0.1 nM.

A Three Ig Domain FGF Receptor and a Two Ig Domain FGF Receptor Mediate Biological Responses to Both Acidic and Basic FGF.

To determine whether any of the membrane spanning forms of the FGF receptor are activated by either aFGF or bFGF, we expressed these receptors in *Xenopus* oocytes and measured receptor activation using a sensitive Ca^{++} efflux assay. This assay has been used to examine expression of receptors for other Ca^{++} mobilizing ligands including cholecystikinin, bombesin, vasopressin, and angiotensin II. Ligand-induced efflux reflects a mobilization of Ca^{++} from intracellular stores, leading to increased levels of intracellular Ca^{++} and accelerated efflux. For our experiments full-length cDNA were transcribed in vitro and the capped mRNAs were injected into *Xenopus* oocytes. After 48 hours, the injected oocytes were loaded with $^{45}\text{CaCl}_2$ and ligand-dependent calcium mobilization was assayed by measuring $^{45}\text{Ca}^{++}$ efflux (Fig. 11). Addition of either aFGF (A and B) or bFGF (C and D) induced a rapid and large efflux of $^{45}\text{Ca}^{++}$ from oocytes injected with RNA encoding the chicken FGF receptor (A and C) or RNA encoding the human h2 receptor (B and D). In contrast, oocytes injected with either human h3 RNA (B and D) or water alone (A-D) showed no response to either aFGF or bFGF. As a positive control, carbachol was added following the 100 minute timepoint. Oocytes express endogenous receptors for carbachol, and oocytes injected with either FGF receptor RNA or water exhibited a positive response after carbachol stimulation. We conclude that both the 3 Ig domain form (cFGF-R) and the 2 Ig domain form (h2) of the FGF receptor are biologically responsive to both acidic and basic FGF. Thus, the ligand binding domains for acidic and basic FGF appear to lie in the receptor region encompassing the highly acidic domain and the IgII and IgIII domains.

While the human h2 receptor clearly responds to both ligands, no response was seen in oocytes injected with RNA encoding the h3 receptor form. It is possible that the three amino acid differences between h2 and h3 cause these proteins to respond differently. Alternatively, the lack of a response in oocytes injected with the h3 RNA may be due to unusually low expression levels of the h3 protein. Unfortunately, we have not yet been able to determine receptor protein expression levels in oocytes.

FGF-R forms having either 2 or 3 extracellular Ig-like domains will bind and respond to both acidic and basic FGF. Some forms of FGF receptor mRNA encode only the extracellular domain of the FGF receptor, a protein that is
5 likely to be secreted from the cell.

The fact that a 2 Ig-like domain form of the FGF receptor (h2) binds both aFGF and bFGF with high affinity has allowed us to localize the binding domains for these ligands to a region encompassing the highly acidic region and the IgII and
10 IgIII domains.

The h4 and h5 receptor forms lack transmembrane sequences and presumably represent secreted forms of the FGF receptor. Preliminary data indicates that cells transfected with the h4 cDNA secrete a 70 kd protein which is recognized by
15 anti-FGF-R polyclonal antisera.

The role of secreted forms of the FGF receptor is unclear. The secreted forms may act to regulate levels of extracellular FGFs, and thereby regulate availability of FGFs to cell surface FGF receptors. Alternatively, the secreted FGF
20 receptors may serve to store and sequester FGFs at a particular location. Another possibility is that the secreted forms may bind to FGFs in an intracellular compartment and subsequently serve as a means for secreting the factor. This is an important consideration in view of the fact that aFGF and bFGF
25 do not contain signal sequences and their mechanism of secretion is unknown.

Our results suggest that receptor diversity can be generated by alternative splicing. We have isolated a total of 5 different FGF receptor cDNA species. Comparison of amino
30 acid sequences strongly indicates that all 5 species are derived from the same gene. Another interesting feature of the human receptor forms is the presence or absence of the ArgMet sequence (amino acids 59 and 60 in h2 and h4) in the extracellular domain.

Affinity labeling experiments using either ^{125}I -aFGF
35 or ^{125}I -bFGF identified a single 145 kd receptor protein on transfectant cells expressing the 3 Ig domain form of the FGF receptor, and a single 125 kd receptor protein on transfectant

cells expressing the 2 domain form of the FGF receptor (see Fig. 11). It is possible that the presence of two receptor species may reflect coexpression of the 3 Ig domain and 2 Ig domain forms of the receptor. Our data clearly establish that a single FGF receptor species can bind both aFGF and bFGF with high affinity and mediate the biological effects of these factors. We have used acidic and basic FGF in these experiments because they are the best characterized members of the FGF family, and are readily available in recombinant form.

Example 6

Competitive Binding of FGF-R Peptides or Fragments, Development of FGF-R Related Antagonists or Agonists

A fragment containing all or part of the extracellular, ligand-binding domain of the FGF-R (i.e., containing amino acids 22-374 of Figure 3 or 22-285 of Figure 4) or analogs thereof are expressed in a host (e.g. mammalian cells or baculovirus infected insect cells) and purified as described in Example 1. Alternatively, fragments of the ligand-binding domain are made using a peptide synthesizer (Applied Biosystems) and purified by HPLC. Different concentrations of the FGF-R fragment or analogs thereof (FGF-Rexs) are tested for their ability to block the binding of ^{125}I -FGF to Swiss 3T3 cells. Competitive binding is performed as described in Figure 1A in Example 1 using FGF-Rexs instead of unlabeled ligand and competitive binding is determined.

FGF-Rexs are also tested for their ability to inhibit FGF-induced mitogenesis as measured by ^3H -thymidine incorporation into cells and by counting cell numbers. FGF-Rexs which block binding of FGF to the cell-surface receptor may act as an antagonist and block ^3H -thymidine uptake and the increase in cell number induced by FGF. FGF-Rexs may also act as agonists, i.e. by dimerization with the cell surface receptor which may mimic a ligand-mediated receptor-receptor interaction. In such an instance, FGF-Rexs may stimulate mitogenesis in the absence of ligand or may enhance the FGF mediated mitogenic response.

FGF-Rexs are also tested for their ability to inhibit

or activate FGF-induced tyrosine phosphorylation of the 90 substrate protein in Swiss 3T3 cells or autophosphorylation of the cell-associated FGF-R. FGF-Rexs which block FGF-induced tyrosine phosphorylation are antagonists. FGF-Rexs which
5 activate autophosphorylation of the cell-associated FGF-R in the absence of FGF are agonists.

FGF-Rexs are also tested for their anti-angiogenic activity. FGF-Rexs are tested first for their ability to inhibit the FGF-induced growth and the mobilization of
10 endothelial cells into vessels in vitro. Angiogenesis is assayed in vitro using an aortic ring assay. Aortic rings are placed in a collagen matrix formed in the presence or absence of FGF and FGF-Rexs. Endothelial cells sprout and form vessels from the aortic ring within a few days in the presence of FGF.
15 The addition of FGF-Rexs which are antagonists in the previous assays inhibit the FGF-induced growth of capillary sprouts. FGF-Rexs which are angiogenic even in the absence of FGF are agonists.

FGF analogs, angiogenic factors, anti-angiogenic
20 factors as well as antibodies to the extracellular portion of the FGF-R are tested for their ability to bind directly or compete for binding of native FGF for binding to purified or expressed FGF-R. In addition, they are tested for their ability to stimulate mitogenesis (agonists) or inhibit FGF-
25 dependent mitogenesis (antagonists) as well as tyrosine phosphorylation in cells expressing the FGF-R. These studies are important in determining if the mode of action of each angiogenic and anti-angiogenic factor, etc., is receptor-mediated and in determining if there is receptor specificity
30 (i.e. acidic versus basic FGF-R) for angiogenic and anti-angiogenic factors.

FGF analogs are radiolabeled and binding is performed with labeled ligand, purified or expressed receptor in the appropriate physiologic buffer (i.e. culture media or phosphate
35 buffered saline (PBS)) for 0.5 at 37°C or 2-24 hrs at 4°C. The complex is precipitated (5-10% polyethylene glycol, 1 mg/ml IgG) and separated by filtration through filters (i.e. Whitman GFA) and the associated radioactivity determined.

While the invention has been described in connection with certain specific embodiments thereof, it should be recognized that various modifications as may be apparent to one of skill in the art to which the invention pertains also fall
5 within the scope of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A method for modifying in vivo a fibroblast growth factor receptor modulated activity comprising administering to
5 a patient an amount of a fibroblast growth factor receptor blocking agent effective to inhibit fibroblast growth factor binding to said fibroblast growth factor receptor.
2. A method of claim 1, wherein the agent comprises a human fibroblast growth factor receptor or a portion thereof.
- 10 3. A method of claim 2, wherein the human fibroblast growth factor receptor or portion thereof is produced in a cell transformed with a nucleic acid containing at least about 15 bases of a sequence selected from the group consisting of:
 - a) a DNA sequence in Figures 3 or 4;
 - 15 b) a DNA sequence encoding a polypeptide of Figures 3, 4 or 7; and
 - c) a DNA sequence substantially homologous to a sequence of Figures 3 or 4.
4. A method of claim 2, wherein the blocking agent
20 comprises a fibroblast growth factor receptor extracellular domain without a tyrosine kinase region.
5. A method of inhibiting binding between a fibroblast growth factor and a fibroblast growth factor receptor present in a solution, said method comprising a step of adding a
25 fibroblast growth factor receptor peptide to said solution.
6. A method of claim 5, wherein said fibroblast growth factor receptor peptide is homologous to a sequence described in Figures 3, 4 or 7.
7. A composition comprising a soluble polypeptide having
30 between about five and two hundred contiguous amino acids from a human fibroblast growth factor receptor extracellular domain.
8. A composition according to claim 7, wherein the polypeptide comprises an IgII or an IgIII domain.
9. A composition according to claim 8, wherein the
35 extracellular domain comprises about 80 amino acids from residues 1 to 287 of a human fibroblast growth factor receptor Figure 7.
10. A composition according to claim 7, wherein the IgII

domain comprises about 7 amino acids from residues 85 to 141 of a human sequence of Figure 7.

11. A composition according to claim 6, wherein the polypeptide comprises both an IgII and an IgIII domain.
- 5 12. A composition according to claim 11, wherein the polypeptide further comprises a carboxy terminal sequence substantially homologous to the 79 amino acid sequence from residues 222 to 300 of a soluble human protein of Figure 7.
- 10 13. A composition according to claim 12 consisting essentially of h4 or h5.
14. A composition comprising a substantially pure polypeptide of less than about 85 KDa comprising a fibroblast growth factor-binding domain of a fibroblast growth factor receptor.
- 15 15. A composition according to claim 14, wherein said polypeptide is soluble.
16. A composition according to claim 14, wherein said polypeptide further comprises a segment selected from the group consisting of a signal segment, an IgI segment, an acidic
20 segment, an IgII segment, an IgIII segment, an IgIIIT segment, and a transmembrane segment.
17. A composition according to claim 14, wherein said polypeptide is homologous to a sequence described in Figures 3, 4 or 7.
- 25 18. A composition according to claim 17, wherein the polypeptide is present in a multi-chain complex of proteins.
19. A composition according to claim 14, wherein the fibroblast growth factor-binding domain includes at least about 30 amino acids of each of both IgII and IgIII domains.
- 30 20. A composition according to claim 14, wherein the fibroblast growth factor receptor is a chicken fibroblast growth factor receptor.
21. An isolated nucleic acid encoding a human fibroblast growth factor receptor which substantially lacks an
35 intracellular domain.
22. An isolated nucleic acid of claim 21, wherein said receptor comprises a sequence homologous to an IgII domain described in Figure 7.

23. An isolated nucleic acid encoding a substantially full length IgII domain of a fibroblast growth factor receptor.

24. An isolated nucleic acid of claim 23, further encoding at least one additional peptide segment selected from the group consisting of a signal segment, an IgI segment, an acidic segment, an IgIII segment, an IgIIIT segment, a transmembrane segment, and a tyrosine kinase segment.

25. An isolated nucleic acid of claim 24, wherein said additional sequence is homologous to a corresponding sequence described in Figures 3, 4 or 9.

26. An isolated nucleic acid of claim 23, wherein said IgII peptide sequence is native to a human.

27. An isolated nucleic acid of claim 23, further comprising a transcription promoter sequence.

28. An isolated nucleic acid encoding a soluble human fibroblast growth factor receptor.

29. An isolated nucleic acid of claim 28, wherein said soluble human growth factor receptor is homologous to h4 or h5.

30. A protein made by expressing an isolated nucleic acid of claim 29.

31. A composition comprising a fibroblast growth factor receptor segment made by expressing an isolated nucleic acid of claim 21, 23, or 28.

32. A method for making a protein comprising a fibroblast growth factor receptor segment, said method comprising expressing an isolated nucleic acid of claim 21, 23, or 28.

33. A composition comprising a nucleic acid according to claim 21, 23, or 28, attached to a reporter molecule.

34. A method for making a fibroblast growth factor receptor peptide, wherein the peptide is produced in a cell transformed with a nucleic acid containing at least about 20 bases of a sequence selected from the group consisting of:

a) a DNA sequence in Figures 3, 4 or 9;

b) a sequence encoding a polypeptide of Figures 3, 4 or 7; and

c) a sequence substantially homologous to a sequence of Figures 3, 4 or 9.

35. A method for producing an antibody against a

fibroblast growth factor receptor fragment, said method comprising a step of producing an antibody against a polypeptide epitope homologous to a sequence of at least six contiguous amino acids described in Figures 3, 4 or 7.

5 36. A method of claim 35, wherein said epitope is selected from the group of protein segments consisting of a signal segment, an IgI segment, an acidic segment, an IgII segment, an IgIII segment, and an IgIIIT segment.

10 37. A method of measuring a fibroblast growth factor or a fibroblast growth factor receptor in a target sample, said method comprising the steps of:

combining said target sample with a fibroblast growth factor receptor segment; and
15 determining the extent of binding between said segment and said sample.

38. A transformed cell capable of expressing a polypeptide homologous to at least a portion of a fibroblast growth factor receptor.

20 39. A transformed cell as in claim 38, wherein the polypeptide is homologous to substantially the entire membrane bound form of a human fibroblast growth factor receptor.

40. A transformed cell as in claim 38, wherein the polypeptide is homologous to substantially the entire soluble form of a human fibroblast growth factor receptor.

25 41. A transformed cell as in claim 40, wherein the cell is capable of secreting the human fibroblast growth factor receptor.

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INHIBITION OF 125 I- LABELED bFGF BINDING (%)

FIG. 1.A

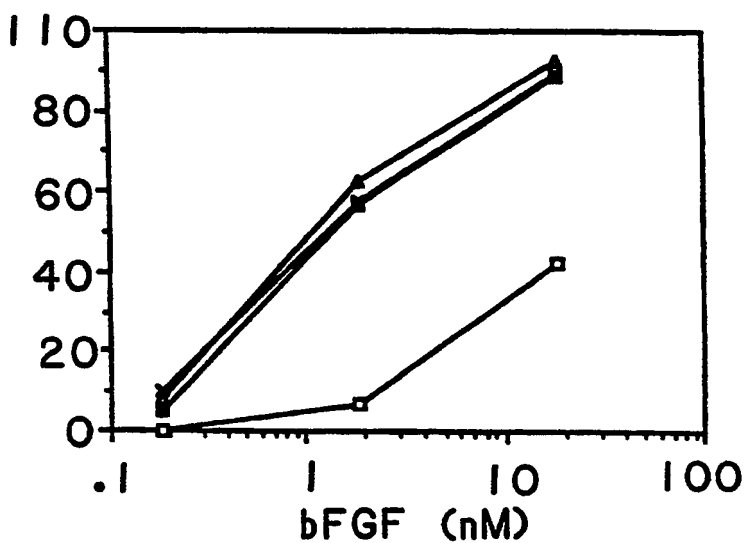
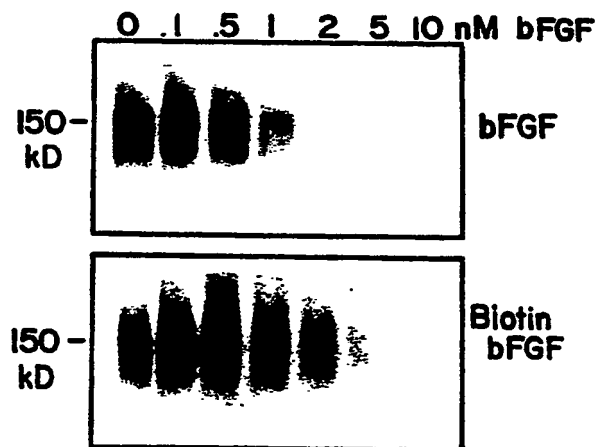


FIG. 1.B



SUBSTITUTE SHEET.

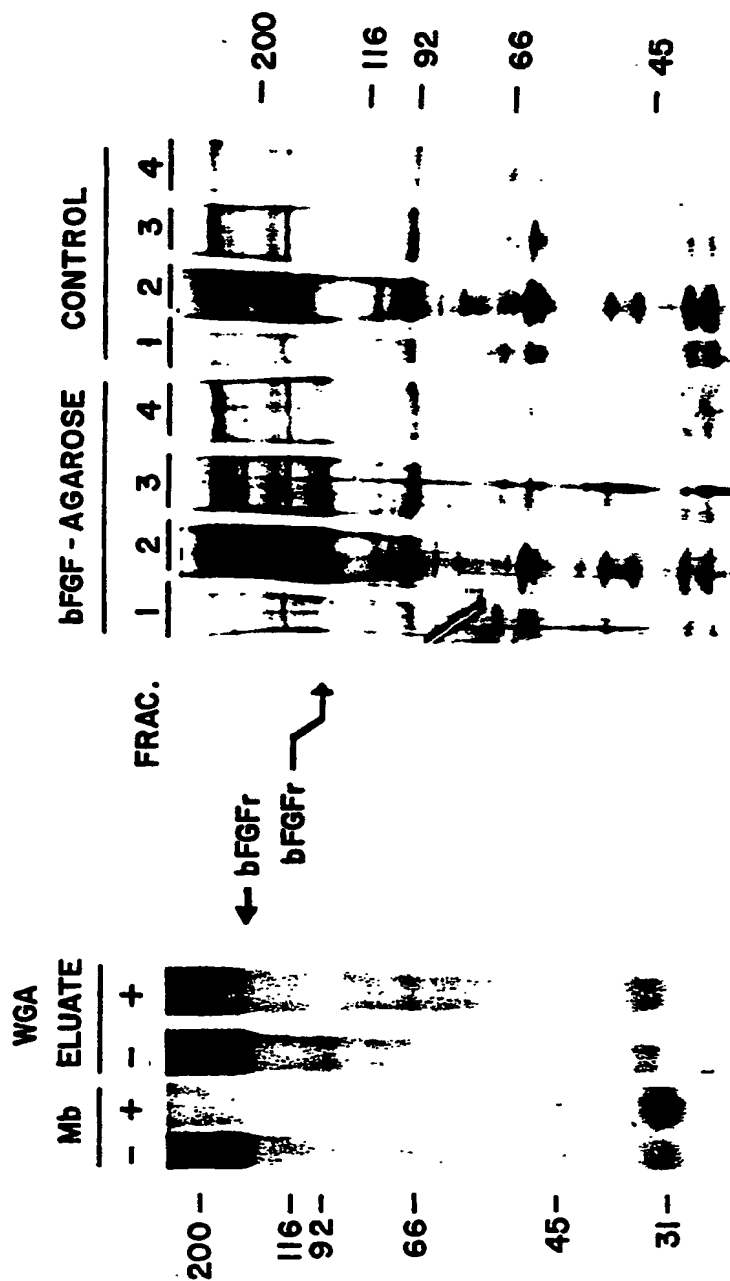


FIG. 2.B

FIG. 2.A

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FIG. 3.FIG. 3A. FIG. 3B.
FIG. 3C. FIG. 3D.

FIG. 3A.

1 GGGGACCGGGCGGTGCGGGCAGCGCTGAGCGGCGGCCGTGGGGGGGACCGCGCGCTCCG
 120 GCGGGCGGCGGGGGGCTGCGCTCTCGCCCGGCGGGGGCTCCCTCCATTGTTCCGGGG
 239 GCCCCATGGAGGGGGCGGTTGAGCGCAGTCGCTGAGCAGTAGCCGCAGCAGTGGG Met
 30
 341 Thr Leu Ser Ala Ala Arg Pro Ala Pro Thr Leu Pro Asp Gln Ala
 20 30
 431 Gly Asp Leu Leu Gln Leu Arg Cys Arg Leu Arg Asp Asp Val Gln
 50 60
 521 Arg Thr Arg Ile Thr Gly Glu Glu Val Glu Val Arg Asp Arg Val
 80 90
 611 Gly Ser Glu Thr Thr Tyr Phe Ser Val Asn Val Ser Asp Ala Leu
 110 120
 701 Glu Lys Glu Ala Asp Asn Thr Lys Pro Asn Gln Ala Val Ala Pro
 140 150
 791 Pro Ala Ala Lys Thr Val Lys Phe Lys Cys Pro Ser Gly Gly Thr
 170 180
 881 Pro Asp His Arg Ile Gly Gly Tyr Lys Val Arg Tyr Ala Thr Trp
 200 210
 971 Thr Cys Ile Val Glu Asn Lys Tyr Gly Ser Ile Asn His Thr Tyr
 230 240
 1061 Ala Gly Leu Pro Ala Asn Lys Thr Val Ala Leu Gly Ser Asn Val
 260 270
 1151 Trp Leu Lys His Ile Glu Val Asn Gly Ser Lys Ile Gly Pro Asp
 290 300
 1241 Thr Asp Lys Glu Me Glu Val Leu His Leu Arg Asn Val Ser Phe
 320 330
 1331 Ile Ser His His Ser Ala Trp Leu Thr Val Leu Glu Ala Thr Glu
 350 360
 1421 Ile Tyr Cys Thr Gly Ala Phe Leu Ile Ser Cys Met Val Val Thr
 380 390
 ATT TAC TGC ACC GGC GCC TTC CTC ATC TCC TGC ATG GTG GTG ACA

SUBSTITUTE SHEET

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FIG. 3B.

GGGAGCGGCTCCCGCCGCCACCTCGGGGCGGGGTCGGCCATGGGGCGGGCGGAGTGAGG
 AGCGGCGGGCAGCGCGCCTCCGCCGGCCCGGCGGGGACTCGGCTCAGAGCGGGCGAGCGG

Phe	Thr	Trp	Arg	Cys	Leu	Ile	Leu	Trp	Ala	Val	Leu	Val	Thr	Ala
TTT	ACC	TGG	AGG	TGC	CTC	ATC	CTT	TGG	GCT	GTG	CTG	GTC	ACA	GCC
10														
Leu	Pro	Lys	Ala	Asn	Ile	Glu	Val	Glu	Ser	His	Ser	Ala	His	Pro
CTG	CCC	AAA	GCG	AAC	ATC	GAG	GTG	GAG	TCC	CAC	TGG	GCG	CAC	CCG
40														
Ser	Ile	Asn	Trp	Val	Arg	Asp	Gly	Val	Gln	Leu	Pro	Glu	Asn	Asn
AGC	ATC	AAC	TGG	GTG	CGT	GAT	GGA	GTG	CAG	CTG	CCC	GAG	AAC	AAC
70														
Pro	Glu	Asp	Ser	Gly	Leu	Tyr	Ala	Cys	Met	Thr	Asn	Ser	Pro	Ser
CGC	GAG	GAC	TGC	GGG	CTC	TAT	GCC	TGC	ATG	ACC	AAC	AGC	CCC	TGC
100														
Pro	Ser	Ala	Glu	Asp	Asp	Asp	Asp	Glu	Asp	Asp	Ser	Ser	Ser	Glu
CCT	TCT	GCA	GAG	GAT	GAT	GAT	GAT	GAA	GAT	GAT	TCC	TCC	TCC	GAG
130														
Tyr	Trp	Thr	Tyr	Pro	Glu	Lys	Met	Glu	Lys	Lys	Leu	His	Ala	Val
TAC	TGG	ACC	TAT	CCC	GAG	AAG	ATG	GAG	AAG	AAG	CTG	CAT	GCC	GTC
160														
Pro	Asn	Pro	Thr	Leu	Arg	Trp	Leu	Lys	Asn	Gly	Lys	Glu	Phe	Lys
CGC	AAC	CCC	ACG	CTG	CGC	TGG	CTG	AAG	AAC	GGC	AAG	GAG	TTC	AAG
190														
Ser	Ile	Ile	Met	Asp	Ser	Val	Val	Pro	Ser	Asp	Lys	Gly	Asn	Tyr
AGC	ATC	ATC	ATG	GAC	TGG	GTG	GTG	CCA	TCA	GAT	AAG	GGC	AAC	TAC
220														
Gln	Leu	Asp	Val	Val	Glu	Arg	Ser	Pro	His	Arg	Pro	Ile	Leu	Gln
CAG	CTG	GAT	GTC	GTG	GAG	CGC	TCC	CCG	CAT	CGG	CCC	ATC	CTG	CAG
250														
Glu	Phe	Val	Cys	Lys	Val	Tyr	Ser	Asp	Pro	Gln	Pro	His	Ile	Gln
GAG	TTT	GTC	TGC	AAG	GTG	TAC	AGC	GAG	CCG	CAG	CCC	CAC	ATC	CAG
280														
Asn	Leu	Pro	Tyr	Val	Gln	Ile	Leu	Lys	Thr	Ala	Gly	Val	Asn	Thr
AAC	TTG	CCC	TAC	GTG	CAG	ATC	CTG	AAG	ACC	GCT	GGC	GTT	AAC	ACG
310														
Glu	Asp	Ala	Gly	Glu	Tyr	Thr	Cys	Leu	Ala	Gly	Asn	Ser	Ile	Gly
GAG	GAT	GCT	GGG	GAG	TAT	ACA	TGT	TTG	GCG	GGT	AAT	TCT	ATT	GGG
340														
Gln	Ser	Pro	Ala	Met	Met	Thr	Ser	Pro	Leu	Tyr	Leu	Glu	Ile	Ile
CAG	TCA	CCA	GCC	ATG	ATG	ACG	TCC	CCC	CTC	TAC	CTG	GAG	ATC	ATC
370														
Val	Ile	Ile	Tyr	Lys	Met	Lys	Ser	Thr	Thr	Lys	Lys	Thr	Asp	Phe
GTC	ATC	ATC	TAC	AAG	ATG	AAG	AGC	ACC	ACC	AAG	AAG	ACA	GAC	TTC
400														

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410
 1511 Asn Ser Gln Leu Ala Val His Lys Leu Ala Lys Ser Ile Pro Leu
 AAC AGC CAG CTG GCC GTG CAC AAG CTG GCC AAG AGC ATC CCA CTG
 440
 1601 Ser Gly Val Met Leu Val Arg Pro Ser Arg Leu Ser Ser Ser Gly
 TCG GGT GTG ATG TTG GTG CGG CCC TCA CGG CTC TCC TCC AGC GGA
 470
 1691 Pro Arg Trp Glu Leu Pro Arg Asp Arg Leu Ile Leu Gly Lys Pro
 CCG CGC TGG GAG CTG CCA CGG GAC AGG CTG ATC CTG GGC AAG CCG
 500
 1781 Gly Leu Asp Lys Asp Lys Pro Asn Arg Val Thr Lys Val Ala Val
 GGC CTG GAC AAG GAC AAG CCA AAC CGC GTC ACC AAA GTG GCT GTA
 530
 1871 Ile Ser Glu Met Glu Met Met Lys Met Ile Gly Lys His Lys Asn
 ATC TCC GAG ATG GAG ATG ATG AAG ATG ATC GGC AAG CAC AAG AAC
 560
 1961 Val Ile Val Glu Tyr Ala Ser Lys Gly Asn Leu Arg Glu Tyr Leu
 GTC ATC GTG GAG TAC GCC AGC AAA GGC AAC CTG CGT GAG TAC CTG
 590
 2051 Arg Ile Pro Glu Glu Gln Leu Ser Phe Lys Asp Leu Val Ser Cys
 CGC ATC CCC GAG GAG CAG CTC TCC TTC AAG GAC CTG GTG TCC TGC
 620
 2141 Cys Ile His Arg Asp Leu Ala Ala Arg Asn Val Leu Val Thr Glu
 TGC ATC CAC AGG GAC CTG GCC GCC AGG AAC GTG CTG GTG ACC GAG
 650
 2231 His His Ile Asn Tyr Tyr Lys Lys Thr Thr Asn Gly Arg Leu Pro
 CAC CAC ATC GAT TAC TAC AAG AAG ACC ACA AAC GGC CGC TTG CCG
 680
 2321 His Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu Trp Glu Ile
 CAT CAG AGT GAT GTT TGG TCC TTC GGT GTG CTG CTG TGG GAG ATC
 710
 2411 Leu Phe Lys Leu Leu Lys Glu Gly His Arg Met Asp Lys Pro Ser
 CTC TTC AAG CTG CTG AAG GAA GGC CAC AGG ATG GAC AAG CCC AGC
 740
 2501 Ala Val Pro Ser Gln Arg Pro Thr Phe Lys Gln Leu Val Glu Asp
 GCC GTG CCC TCC CAG CGC CCC ACC TTC AAG CAG CTG GTG GAG GAC
 770
 2591 Leu Ser Val Pro Leu Asp Gln Tyr Ser Pro Gly Phe Pro Ala Thr
 CTG TCG GTG CCG TTG GAT CAG TAC TCG CCC GGC TTC CCG GCC ACG
 800
 2681 Asp Pro Leu Pro Asp Glu Pro Cys Leu Pro Arg Cys Pro His
 GAC CCG CTG CCC GAC GAG CCC TGC CTG CCG CGC TGC CCC CCG CAC
 810
 2777 CCGGGCACCACCGCAGGGAAGCTTTCGGCTGCTGTTGGGCTGTTGGT
 2897 CCACGTGGCGGTGCCGCCGTGTCCTATGGGGCCGATGCGCGCTGTGAGCATCGCATCC
 3017 CGCTAGGACAGAAGTCCC GTGTACATAGCTAAAATATGTATAAATATGAATATATATT
 3137 GCTGGTAGATATCAGTTGCTATATATAAAAAAAAAA

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FIG. 3C.

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FIG. 3D.

Arg Arg Gln Val Thr Val Ser Ala Asp 430 Ser Ser Ser Ser Met Asn
 CGC AGA CAG GTA ACA GTG TCA GCA GAT TCC AGC TCC TCC ATG AAC
 Thr Pro Met Leu Ala Gly Val Ser 460 Tyr Glu Leu Pro Glu Asp
 ACC CCC ATG CTG GCC GGC GTC TCC GAG TAT GAG CTG CCC GAG GAC
 Leu Gly Glu Gly Cys Phe Gly Gln 490 Val Val Leu Ala Glu Ala Ile
 CTG GGA GAA GGC TGC TTT GGG CAG GTG GTG CTC GCG GAG GCC ATC
 Lys Met Leu Lys Ser Asp Ala Thr 520 Glu Lys Asp Leu Ser Asp Leu
 AAG ATG CTC AAG TCC GAT GCC ACA GAG AAG GAC CTG TCC GAC CTC
 Ile Ile Asn Leu Leu Gly Ala Cys 550 Thr Gln Asp Gly Pro Leu Tyr
 ATC ATC AAC CTG CTG GGT GCC TGC ACG CAG GAC GGG CCC CTC TAT
 Gln Ala Arg Arg Pro Pro Gly Met 580 Glu Tyr Cys Tyr Asn Pro Thr
 CAG GCA CCG CCG CCA CCG GGC ATG GAG TAC TGC TAC AAC CCC ACA
 Ala Tyr Gln Val Ala Arg Gly Met 610 Glu Tyr Leu Ala Ser Lys Lys
 GCG TAC CAG GTG GCA CCG GGC ATG GAG TAC CTG GCC TCC AAA AAG
 Asp Asn Val Met Lys Ile Ala Asp 640 Phe Gly Leu Ala Arg Asp Ile
 GAC AAC GTG ATG AAG ATC GCT GAC TTC GGG CTG GCC CCG GAC ATC
 Val Lys Trp Met Ala Pro Glu Ala 670 Leu Phe Asp Arg Ile Tyr Thr
 GTG AAG TGG ATG GCC CCG GAG GCT CTG TTC GAC CGA ATA TAC ACC
 Phe Thr Leu Gly Gly Ser Pro Tyr 700 Pro Gly Val Pro Val Glu Glu
 TTC ACG CTG GGC GGT TCG CCC TAC CCC GGC GTG CCC GTG GAG GAG
 Asn Cys Thr Asn Glu Leu Tyr Met 730 Met Met Arg Asp Cys Trp His
 AAC TGC ACC AAC GAG CTG TAC ATG ATG ATG CCG GAC TGC TGG CAC
 Leu Asp Arg Ile Val Ala Met Thr 760 Ser Asn Gln Glu Tyr Leu Asp
 CTG GAC AGG ATC GTG GCC ATG ACC TCC AAT CAG GAG TAC CTG GAC
 Arg Ser Ser Thr Cys 790 Ser Ser Gly Glu Asp Ser Val Phe Ser His
 GGC AGC TCC ACC TGC TCC TCG GGG GAG GAC TCG GTG TTC TCC CAC
 Ser His Gly Ala Leu Lys Arg 819 His OP
 AGC CAC GGA GCG CTG AAG CCG CAC TGA GGCTCCGCACGCAGCTGTGCCCCC

CGGCTCTTTTTTTTTTATCACCCATTTAAACCCTTCCACGAGGTCTGTGCTTGACATCC
 CAGCGCTGCCCAACCCACACGCTGTGGGGTGTGCAGCACACGGGGCCGCCCGGGGATCAG
 ACA TGTCTTTTAAAGGGTGGTTACCAGAGCTGTGCCAGGCTGGTAGGGAGGTGCTGGTG

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1	GGGGAAGCCAAGGACTTTTCTCGGCTCGAGCTCGGGGGGCC	42
43	CGGCACCGGGACGGTACCCGTGCTGCAGTCGGGCACGCCGG	84
85	GGCCCGCGGGGGGCGCTCGGACGGGCGATGGAGCCGGTCTGCA	126
127	AGGAAAGTGAGGCGCCGCCGCTGCGTTCTGGAGGAGGGGGG	168
169	ACAAGGTCTGGAGACCCCGGGTGGCGGACGGGAGCCCTCCCG	210
211	CGCCCGCGCCTCGGGGGCAGGCTCGGGCTCCATTGTTCCC	252
253	GGCCGGGCTGGAAGGCGCGAGCACCAGCGCCGGCGGGAGTC	294
295	GAGCGCGGGCGCGGAGGACTCTTGCGACCCGGCGAGGACCG	336
337	GAACAGAGCCCGGGGGCGGGCGGGCCGGAGCGGGGAGCGGG	378
379	ACACGGCGGCTCGCACAAGCCACGGCGGACTCTCCGAGGG	420
421	GAACCTCCAGCGGAGCGAGGGTCAGTTTGAAAAGGAGGATC	462
463	GAGCTCACTGTGGAGTATCCATGGAGATGTGGAGCCTTGCA	504
505	CCAACCTCTAACTGCAGAACTGGGATGTGGAGCTGGAAGTGC	546
	I M W S W K C	
547	CTCCTCTTCTGGGCTGTGCTGGTCACAGCCACACTCTGCACC	588
	10 L L F W A V L V T A T L C T	
589	GCTAGGCCGTCCCGACCTTGCGTGAAGAAGATGCTCTCCCG	630
	30 A R P S P T L P E Q D A L P	
631	TCCTCGGAGGATGATGATGATGATGATGACTCCTCTTCAGAG	672
	40 S S E D D D D D D D S S S E	

FIG. 4.1

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673 GAGAAAGAAACAGATAACACCAAACCAACCGCTAGCTCCA 714
E K E T D N T K P N P V A P
715 TATTGGACATCCCCAGAAAAGATGGAAAAGAAATTGCATGCA 756
Y W T S P E K M E K K L H A
757 GTGCCGGCTGCCAAGACAGTGAAGTTCAAATGCCCTTCCAGT 798
V P A A K T V K F K C P S S
799 GGGAGCCCAAACCCACACTGCGCTGGTTGGAAAATGGCAA 840
G T P N P T L R W L E N G K
841 GAATTCAAACCTGACCACAGAATTGGAGGCTACAAGGTCCGT 882
E F K P D H R I G G Y K V R
883 TATGCCACCTGGAGCATCATAATGGACTCTGTGGTGGCCTCT 924
Y A T W S I I M D S V V P S
925 GACAAGGGCAACTACACCTGCAATTGTGGAGAATGAGTACGGC 966
D K G N Y T C I V E N E Y G
967 AGCATCAACCAACATACCAGCTGGATGTCGTGGAGCGGTCC 1008
S I N H T Y Q L D V V E R S
1009 CCTCACC GGCCCATCCTGCAAGCAGGGTTGCCCGCCAACAA 1050
P H R P I L Q A G L P A N K
1051 ACAGTGGCCCTGGGTAGCAACGTGGAGTTTCATGTGTAAGGTG 1092
T V A L G S N V E F M C K V
1093 TACAGTGACCGGCAGCGGCACATCCAGTGGCTAAAGCACATC 1134
Y S D P Q P H I Q W L K H I
1135 GAGGTGAATGGGAGCAAGATTGGCCAGACAACCTGCCTTAT 1176
E V N G S K I G P D N L P Y
1177 GTCCAGATCTTGAAGACTGCTGGAGTTAATACCACCGACAAA 1218
V Q I L K T A G V N T T D K
1219 GAGATGGAGGTGCTTCACTTAAGAAATGTCTCCTTTGAGGAC 1260
E M E V L H L R N V S F E D
1261 GCAGGGGACTATAGGTGCTTGGCGGGTAACCTCTATCGGACTC 1302
A G E Y T C L A G N S I G L
1303 TCCCATCACTCTGCATGGTTGACCGTTCTGGAAGGCCTGGAA 1344
S H H S A W L T V L E A L E
1345 GAGAGGGCGGCAGTGATGACCTCGCCCTGTACCTGGAGATC 1386
E R P A V M T S P L Y L E I
1387 ATCATCTATTGCACAGGGGCTTCTCACTCGTGCATGGTG 1428
290 300

FIG. 4.2

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I I Y C T G A F L I S C M V 300
1429 GGGTGGGTCATCGTCTACAAGATGAAGAGTGGTACCAAGAAG 1470
G S V I V Y K M K S G T K K 310
1471 AGTGACTTCCACAGCCAGATGGCTGTGCACAAGCTGGCCAAG 1512
S D F H S Q M A V H K L A K 320
1513 AGCATCCCTCTGCGCAGACAGGTAAAGTGTCTGCTGACTCC 1554
S I P L R R Q V I V S A D S 330 340
1555 AGTGCATCCATGAAGTCTGGGGTTCTTCTGTTGCGCATCA 1596
S A S M N S G V L L V R P S 350
1597 GGGCTCTCCTCCAGTGGGAGTCCCATGCTAGCAGGGGTCTCT 1638
R L S S S G T P M L A G V S 360 370
1639 GAGTATGAGCTTCGCGAAGAGCCTCGGCTGGGAGCTGCCTCGG 1680
E Y E L P E D P R W E L P R 380
1681 GACAGACTGGTCTTAGGCAAACCCCTGGGAGAGGGCTGCTTT 1722
D R L V L G K P L G E G C F 390
1723 GGGCAGGTGGTGTGGCAGAGGCTATCGGGCTGGACAAGGAC 1764
G Q V V L A E A I G L D K D 400 410
1765 AAACCGAACCGTGTGACCAAGTGGCTGTGAAGATGTTGAAG 1806
K P N R V T K V A V K M L K 420
1807 TCGGACGCAACAGAGAAAGACTTGTGAGACCTGATCTCAGAA 1848
S D A T E K D L S D L I S E 430 440
1849 ATGGAGATGATGAAGATGATCGGGAAGCATAAGAATATCATC 1890
M E M M K M I G K H K N I 450
1891 AACCTGCTGGGGGCTGCAGCGAGGATGGTCCCTTGTATGTC 1932
N L L G A C T Q D G P L Y V 460
1933 ATCGTGGAGTATGCCTCCAAGGGCAACCTGCGGGAGTAGCTG 1974
I V E Y A S K G N L R E Y L 470 480
1975 CAGGGCGGGAGGGCCCGAGGGCTGGAATACTGCTACAAGCCC 2016
Q A R R P P G L E Y C Y N P 490
2017 AGCCACAACCCAGAGGAGCAGCTCTCCTCCAAGGACCTGGTG 2058
S H N P E E Q L S S K D L V 500 510
2059 TCCTGGGCTACCAAGGTGGCCCGAGGCATGGAGTATCTGGCC 2100
S C A Y Q V A R G M E Y L A 520
2101 TCCAAGAAGTGCATACCCGAGACCTGGCAGCCAGGAATGTC 2142
S K K C I H R D L A A R N V 530

FIG._4.3

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2143 CTGGT⁵⁴⁰GACAGAGGACAATGTGATGAAGATAGCAGACTTTGGC⁵⁵⁰ 2184
L V T E D N V M K I A D F G
2185 CTCGCACGGGACATTCAACCATCGACTACTATAAAAAGACA 2226
L A R D I H H I D Y Y K K T
2227 ACCAACGGGCGACTGCCTGTGAAGTGGATGGCACCAGGCA⁵⁷⁰ 2268
T N G R L P V K W M A P E A⁵⁸⁰
2269 TTATTTGACGGGATCTACACCCAGCAGT⁵⁹⁰GATGTGTGGTCT 2310
L F D R I Y T H Q S D V W S
2311 TTCGGGGTGGTCTGTGGGAGATCTTCACTCTGGGGGGCTCC 2352
F G V L L W E I F T L G G S⁶⁰⁰
2353 CCATACCCGGGTGTGCCTGTGGAGGAAGTTTCAAGCTGCTG⁶¹⁰ 2394
P Y P G V P V E E L F K L L⁶²⁰
2395 AAGGAGGGTCACCGCATGGACAAGCCAGTAAGTGCAGCAAC⁶³⁰ 2436
K E G H R M D K P S N C I N
2437 GAGCTGTACATGATGATGCGGGACTGCTGGCATGCAGTGGCC⁶⁴⁰ 2478
E L Y M M M R D C W H A V P⁶⁵⁰
2479 TCACAGAGACCCAGCTTCAAGCAGCTGGTGGAGACCTGGAC⁶⁶⁰ 2520
S Q R P T F K Q L V E D L D
2521 CGCATCGTGGCCTTGACCTCCAACCAGGAGTACCTGGACCTG⁶⁷⁰ 2562
R I V A L T S N Q E Y L D L
2563 TCCATGCGCGTGGAGCAGTACTCGCCAGCTTTCGGGACACC⁶⁸⁰ 2604
S M P L D Q Y S P S F P D T⁶⁹⁰
2605 CGGAGCTCTACGTGCTCCTCAGGGGAGGATTCCGTCTTCTCT⁷⁰⁰ 2646
R S S T C S S G E D S V F S
2647 CATGAGCGGCTGCCCGAGGAGCCCTGCCCTGCCCGACACCCA⁷¹⁰ 2688
H E P L P E E P C L P R H P⁷²⁰
2689 GCCCAGCTTGCCAATGGCGGACTCAAACGGCGCTGACTGCCA⁷³⁰ 2730
A Q L A N G G L K R R Z
2731 CCCACAGCCCTCGCCAGACTCCACCGTCAGCTGTAACCCTC 2772
2773 ACCCAGAGCCCTGCTGGGCCCACCACCTGTCCGTCCCTGTC 2814
2815 CCCTTTGCTGCTGGCAGGAGCGGGCTGCCTACCAGGGGCTT 2856

FIG. 4.4

3613 GCTGGTGAGCAGGTCGCAAAGGA 3635

FIG. 4.5

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FIG. 5.A

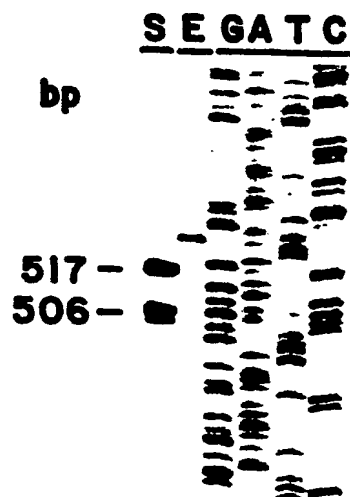


FIG. 5.B

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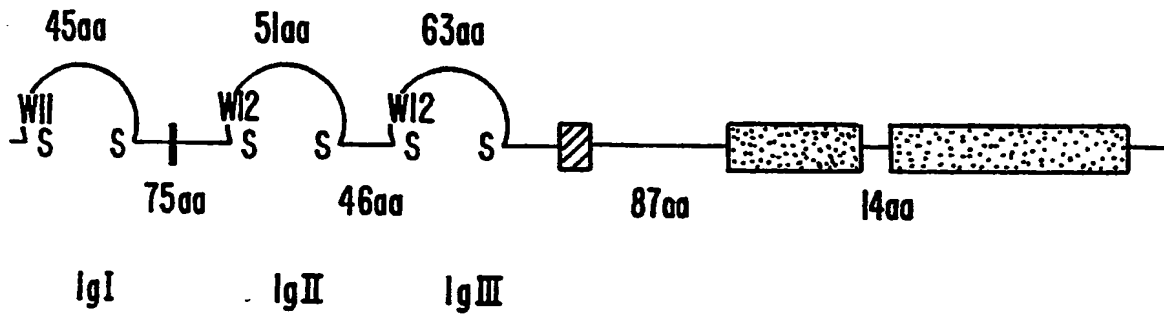


FIG._6.

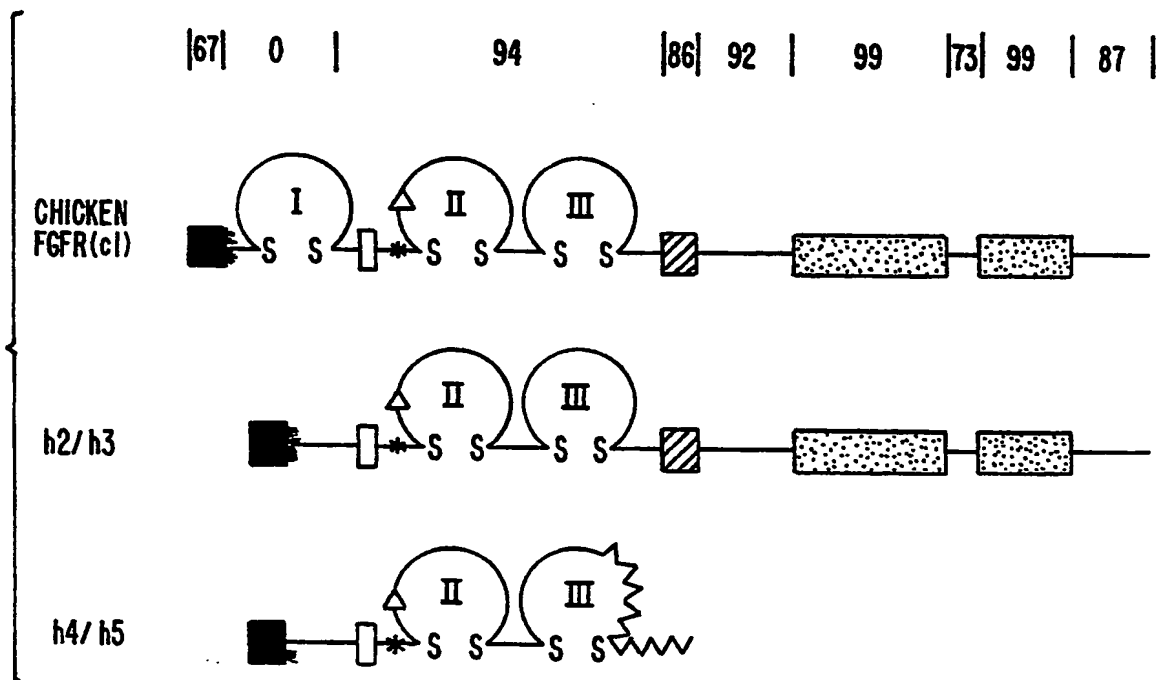


FIG._8.

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CHICKEN FGFR

h2
h3
h4
h5

MFTWRGLILWAVLVATLSAARPA	PTLPDQALPKANIEVESHAHPGDLQLRCRLRDDVOSINWVRDGVQLPEN	75
MWSWKCLIFWAVLVATLCTARPS	SPTLP	30
MWSWKCLIFWAVLVATLCTARPS	SPTLP	30
MWSWKCLIFWAVLVATLCTARPS	SPTLP	30
MWSWKCLIFWAVLVATLCTARPS	SPTLP	30
NRTRITGEEVEVRDRVPEDSGLYACMTNSPSGSETTYFSVNVSDALPSA	EDDDSSSEKEADNTKPNQ-AV	149
-----	-----	62
-----	-----	60
-----	-----	62
-----	-----	60
APYWTYPEKMEKKLHAVPAAKTVKFKCPSGGT	PNPTLRWLKNGKEFKPDHRI	224
APYWTSPERMEKKLHAVPAAKTVKFKCPSGGT	PNPTLRWLKNGKEFKPDHRI	137
APYWTSPERMEKKLHAVPAAKTVKFKCPSGGT	PNPTLRWLKNGKEFKPDHRI	135
APYWTSPERMEKKLHAVPAAKTVKFKCPSGGT	PNPTLRWLKNGKEFKPDHRI	137
APYWTSPERMEKKLHAVPAAKTVKFKCPSGGT	PNPTLRWLKNGKEFKPDHRI	135
NYTCIVENKYGSINHTYQLDVVERS	PHRPILOAGLPANKTVALGNSNVEFVCKVYS	299
NYTCIVENKYGSINHTYQLDVVERS	PHRPILOAGLPANKTVALGNSNVEFVCKVYS	212
NYTCIVENKYGSINHTYQLDVVERS	PHRPILOAGLPANKTVALGNSNVEFVCKVYS	210
NYTCIVENKYGSINHTYQLDVVERS	PHRPILOAGLPANKTVALGNSNVEFVCKVYS	212
NYTCIVENKYGSINHTYQLDVVERS	PHRPILOAGLPANKTVALGNSNVEFVCKVYS	210

FIG. 7.1

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PDNLPYVQILKTAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNSIGISHHSAWLTIVLEATEQSPAMMTSPLYLE	374
PDNLPYVQILKTAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNSIGLSHSAWLTIVLEAEERPAVMTSPLYLE	287
PDNLPYVQILKTAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNSIGLSHSAWLTIVLEAEERPAVMTSPLYLE	285
PDNLPYVQILKVIMAPVFGQSTGKETTVSGAQVPVGRSCPRMGFLLTQAHTLHLSRDLATSPRTSNRGHKVE	287
PDNLPYVQILKVIMAPVFGQSTGKETTVSGAQVPVGRSCPRMGFLLTQAHTLHLSRDLATSPRTSNRGHKVE	285
IIYYCTGAFLLSCNVVTVIIYKMKSTTKKTDNFNSQLAVHKLAKSIPLRROVTVSADSSSMNSGVMLVRPSRLSS	449
IIYYCTGAFLLSCNVGSSVIVYKMKSGTKKSDFHISQMAVHKLAKSIPLRROVTVSADSSISMNSGVLLVRPSRLSS	362
IIYYCTGAFLLSCNVGSSVIVYKMKSGTKKSDFHISQMAVHKLAKSIPLRROVTVSADSSISMNSGVLLVRPSRLSS	360
VSWEQRAAGNGGAGL*	302
VSWEQRAAGNGGAGL*	300
SGTPMLAGVSEYELPEDPRWELPRDRLILGKPLGEGCGQVVLAEAIGLDKDKPNRVTKVAVKMLKSDATEKDLS	524
SGTPMLAGVSEYELPEDPRWELPRDRLVLGKPLGEGCGQVVLAEAIGLDKDKPNRVTKVAVKMLKSDATEKDLS	437
SGTPMLAGVSEYELPEDPRWELPRDRLVLGKPLGEGCGQVVLAEAIGLDKDKPNRVTKVAVKMLKSDATEKDLS	435
DLISEMEMMKMIGKHKNIINLLGACTQDGPYVIVEYASKGNLREYLOARRPPGMEYCYNPTRIPPEQLSFKDLV	599
DLISEMEMMKMIGKHKNIINLLGACTQDGPYVIVEYASKGNLREYLOARRPPGLLEYCYNP[SHN]PEEQLS[KDLV	512
DLISEMEMMKMIGKHKNIINLLGACTQDGPYVIVEYASKGNLREYLOARRPPGLLEYCYNP[SHN]PEEQLS[KDLV	510
SCAYQVARGMEYLASKKCIHRDLAARNVLVTEDNVMKIADFGGLARDIHHIDYYKKTTNGRLPVKWNMAPEALFDRI	674
SCAYQVARGMEYLASKKCIHRDLAARNVLVTEDNVMKIADFGGLARDIHHIDYYKKTTNGRLPVKWNMAPEALFDRI	587
SCAYQVARGMEYLASKKCIHRDLAARNVLVTEDNVMKIADFGGLARDIHHIDYYKKTTNGRLPVKWNMAPEALFDRI	585
YTHQSDVWSFGVLLWEIFTLGGSYPYGPVVEELFKLLKEGHRMDKPSNCTNELYMMMRDCWHAVPSORPTFKQLV	749
YTHQSDVWSFGVLLWEIFTLGGSYPYGPVVEELFKLLKEGHRMDKPSNCTNELYMMMRDCWHAVPSORPTFKQLV	662
YTHQSDVWSFGVLLWEIFTLGGSYPYGPVVEELFKLLKEGHRMDKPSNCTNELYMMMRDCWHAVPSORPTFKQLV	660
EDLDRIVAMTSNOEYLDLSVPLDQYSPGFPATRSSTCSSGSDSVFSHDPLPDEPCLPRCPHSHGALKRH*	819
EDLDRIVAL[TSN]OEYLDLSMPLDQYSP[FPD]TRSSSTCSSGSDSVFSHE[PLPEEPCLPRH]PAQLANGGLKRR[*	733
EDLDRIVAL[TSN]OEYLDLSMPLDQYSP[FPD]TRSSSTCSSGSDSVFSHE[PLPEEPCLPRH]PAQLANGGLKRR[*	731

FIG. 7.2

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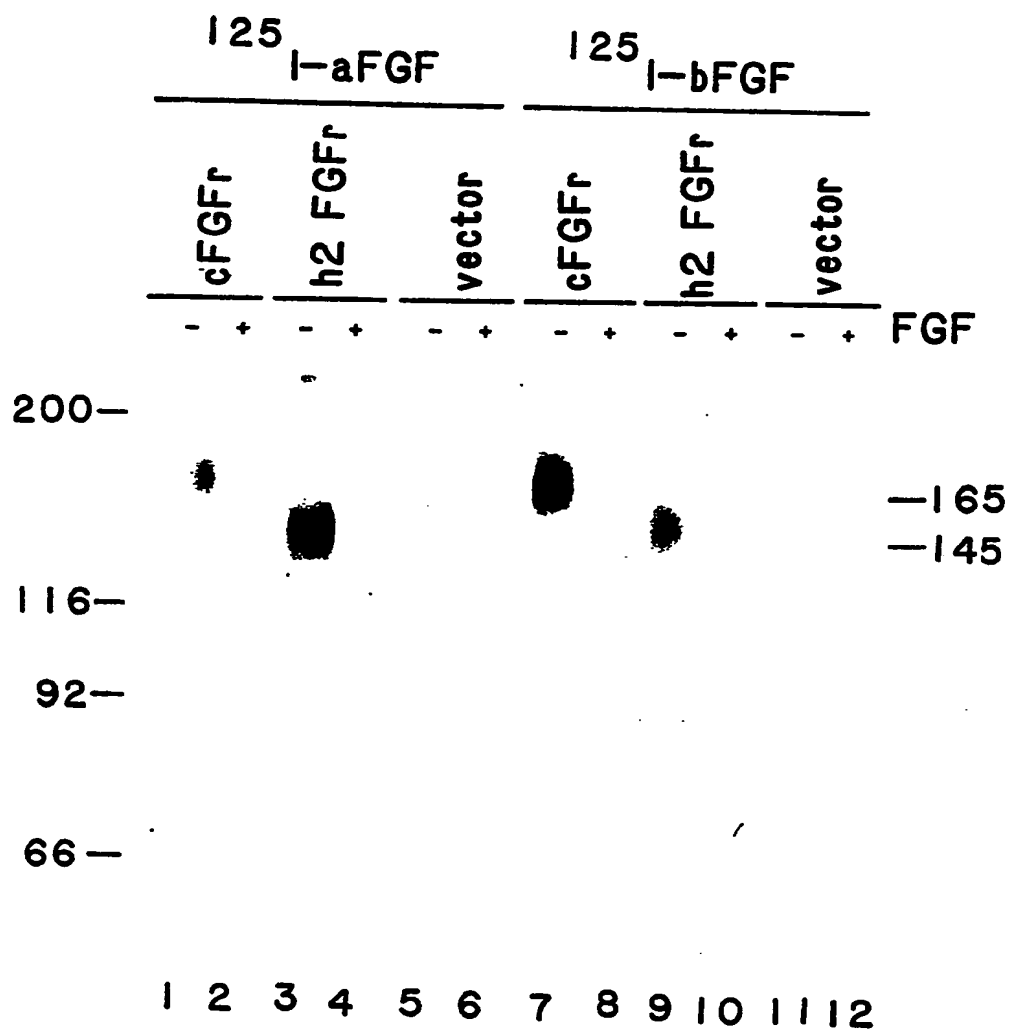


FIG. 10.

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FIG. II. A

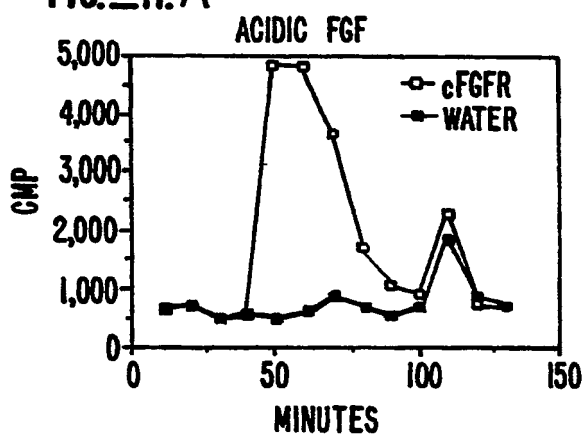


FIG. II. C

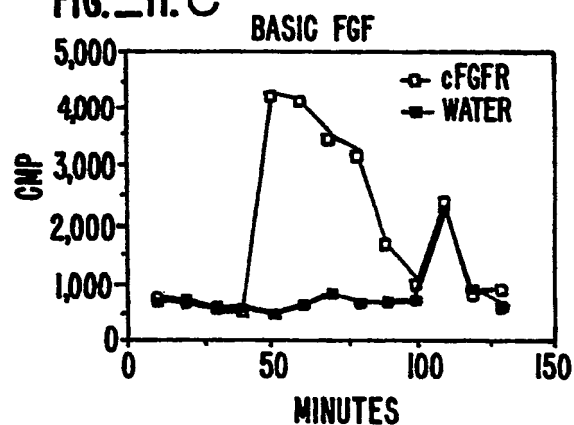


FIG. II. B

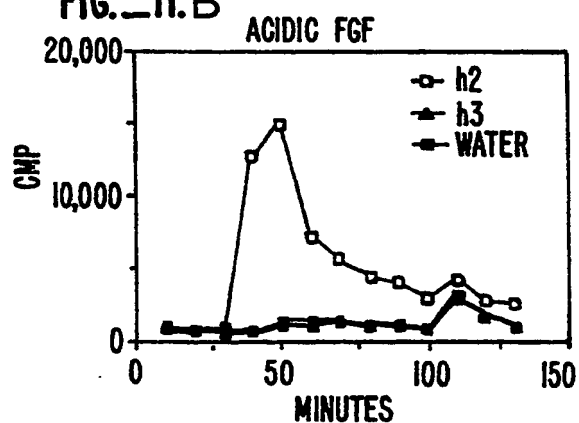
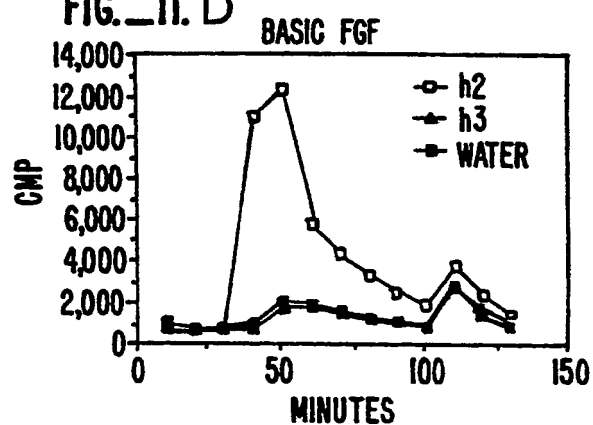


FIG. II. D



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